REVISITING COLD HARDINESS OF PEACH IN GEORGIA

by

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(Under the Direction of Dario J. Chavez)

ABSTRACT

Freezing damage causes major economic losses in peach production in the Southeast U.S. This study analyzed and optimized two methods to measure cold hardiness of peach and provided a better understanding of the freezing process in peach floral buds. For artificial freezing test, floral buds attached to 5 cm stems were the most efficient sampling type to accurately measure cold hardiness. When using DTA, cooling scheme that consisted of an overnight -2 °C incubation before start cooling at 4 °C·h^{-1} improved correlations between LTE temperatures and LT_{50} from standard artificial freezing test. To better understand and localize the freezing damage of flower bud internal structures, we determined their order of cold susceptibility based on vital staining from the least to the most cold resistant as follow: pistils, stamen, corolla and sepal. In conclusion, both traditional and new techniques could accurately measure cold hardiness of peach, and benefit both growers and researchers.

INDEX WORDS: Cold hardiness, peach, artificial freezing test, DTA, cold resistance, LT_{50}, LTE, supercooling, FDA

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DEDICATION

To all the scholars who seek knowledge and dedicated to improving lives. To family and many friends.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW[1]

[1]Liu, J. et al. To be submitted to *The Journal of the American Pomological Society*.

Abstract

Frosts and freezing events can cause major damage to peach production, especially in the Southeast U.S. In Georgia, peach plants start to develop cold hardiness in fall or late summer. This cold hardiness is then lost after the plant's chilling requirement are fulfilled. Most of cold damage observed in Georgia's peach production occurs in spring, when frost coincides with the loss of the plant's cold hardiness. It is important for peach growers to dynamically monitor cold hardiness of peach flower buds to take immediate actions before potential damage could occur. However, the traditional methods for cold hardiness cannot provide both fast and accurate estimations to peach growers to decide the correct freeze protection strategies before a freezing event occurs. This review provides an insight of the freezing process in peach and other fruit trees. It describes the two main causes of freezing damage, as well a description of the defense mechanisms used by plants to survive freezing temperatures. Finally, possible approaches to improve accuracy and efficiency of cold hardiness estimation in peach are also explored.

Peach, *Prunus persica* (L.) Batsch, is an important deciduous fruit. It belongs to the Rosaceous family in which much of the most important commercial fruit crops are found. Peach production ranks 4th among fruit crops worldwide after grape, apple, and pear. In the U.S., California ranked 1st with 607,600 metric tons followed by South Carolina with 68,900 metric tons and Georgia with 40,600 metric tons of peach in 2015 (USDA, 2016).

A series of pre- and post-harvest management procedures ensures tree health, yield, and fruit quality in commercial production. Concomitant to these management procedures, the environment plays an important role. Frosts and freezes can cause major crop losses in the U.S. agriculture more than any other weather event (Snyder and Melo-Abreu, 2005). The study of freeze damage in plants and management techniques used to ameliorate the effect of freezes in fruit production constitute a major area of research.

Freezing process in plants

Plants have a low capacity to maintain their internal temperature and to prevent penetration of freezing environmental temperatures into their tissues. Water is found inside (intracellular) and outside (extracellular) of plant cells. Extracellular water freezes few degrees below 0 °C as temperature decreases. Plants can tolerate the presence of extracellular ice within tissues and still survive (Levitt, 1980). However, plant tissues cannot survive the presence of intracellular ice, which mechanically ruptures cell membranes. Plants can utilize different methods to either avoid intracellular water freezing or tolerate extracellular water freezing inside their tissues. One common way of intracellular ice avoidance is freezing temperature depression. Water is drawn out of the

cells due to the lower water potential of ice when extracellular ice crystals develop (Quamme, 1978). The growth of ice crystals outside the cells and the increase in cell sap concentration lead to a decrease of water potential inside cells. Plant cells thus can attain an equilibrium status when water potential of ice equals to water potential of the intracellular sap. At this point, intracellular water is in thermodynamic equilibrium with extracellular ice and no ice grows inside or outside of cells. During this process, cell sap is concentrated high enough that the solute within the cell functions in the same manner as antifreeze with less water left to freeze. Similarly, freezing temperatures of the cells are depressed by dehydration and are maintained close to the current cell temperature. These cells are free from freezing (Levitt, 1980). When no intracellular ice is formed, the cell protoplasm is not directly exposed to ice with the separation of cell membrane. Cells face mostly the dehydration stress induced by extracellular ice, causing increased cell sap concentration and cell collapse (Levitt, 1980).

There are cases, however, in which freezing events are characterized by a quick drop of temperature. Under these conditions, the rate of intracellular water efflux is not sufficient to maintain the equilibrium. Some plants can still avoid intracellular freezing by another mechanism known as supercooling or undercooling. This mechanism prevents ice formation inside cells even when temperature drops below the solution freezing point, because of the lack of nucleation sites. The phenomenon of supercooling has been observed in many fruit crops, such as peach, pear (*Pyrus communis* L.) and apple (*Malus pumila* Mill.) (Quamme, 1974, 1976, Quamme et al., 1972). Supercooling is most prevalent in xylem and in floral buds among many woody species (George et al., 1982, Levitt, 1980).

Supercooling, as a freezing avoidance mechanism, has its limits. Supercooling of floral buds is achieved by absence of internal nucleators and the presence of a morphological 'barrier' to external nucleators (Ashworth, 1982, Quamme et al., 1995). Tissues can maintain supercooling as long as they are protected against external inoculation and other irritations (e.g. vibration caused by breeze) that are readily capable to trigger the nucleation process under an unstable supercooling state. The extent of supercooling is also limited by the homogenous ice nucleation temperature of water. Water crystalizes spontaneously with or without the presence of nucleation agents when temperature reaches a homogenous ice nucleation point (Ashworth, 1991 Burke et al., 1976), which is a characteristic of water. In the field of plant science, the lowest temperature in which water supercooled to was reported in the xylem of black ash (*Fraxinus nigra* Marsh.) under laboratory conditions as low as -47 °C (George et al., 1974a).

It has been observed that freezing of supercooled water rapidly ruptures cell membranes and causes lethal damage (Ashworth, 1982, Biermann et al., 1979, George et al., 1974b, Ishikawa and Sakai, 1985, Levitt, 1980, Quamme, 1974, Wolf and Pool, 1987). Once nucleation of supercooled water is triggered, the dramatic sudden freezing is far more likely to be lethal than gradual freezing when no supercooling occurs. One example of this was demonstrated by White and Weiser (1964) in white cedar (*Thuja occidentalis* L.). White cedar evergreen foliage was capable of surviving at -87 °C without injury when cooled slowly demonstrating tolerance of extracellular ice formation; however, it was damaged at -10 °C due to the freezing of intracellular water at a rapid cooling rate (Weiser, 1970).

Freezing induces damage to plant tissues in different ways. As mentioned before, plant cells cannot survive the presence of intracellular ice in nature. Only few exceptions under laboratory conditions were observed, when ultrarapid artificial freezing allows only formation of small ice crystals that does not necessarily produce damage (Levitt, 1980, Luyet, 1951). Yet, this type of freezing is not observed in nature. Intracellular ice crystals cause fatal damage to cells by rupturing cell membranes. Dehydration stress can be imposed to plant cells when water freezes extracellularly. Water from cell sap is drawn to ice crystals outside of cells due to low water potential of ice. In this case, injury to the plant is not caused directly by low temperature nor freezing itself, but by the indirect water stress as an effect of extracellular ice. Cells are subjected to water stress with concentrated cell sap and reduced cell sizes which may lead to cell death (Levitt, 1980).

Dormancy

Dormancy is one way deciduous trees survive unfavorable environmental conditions such as low temperature in winter (Faust et al., 1997). Bud dormancy starts with paradormancy from the end of summer to late fall when day length is shorter. During paradormancy, other tissues of the plant detect the environmental cues and send out a physiological signal to buds, therefore induce bud dormancy (Lang et al., 1987). The term ‘paradormancy’ was previously known as ‘correlated inhibition’ due to its nature (Lang et al., 1987). The transition from paradormancy to endodormancy occurs in fall when dormancy is regulated by physiological factors from inside of the bud. This type of dormancy was also known as ‘rest’ in previous literature (Lang et al., 1987). Endodormancy becomes progressively deeper afterward, and is further divided into deep

endodormancy (d-endodormancy) and shallow endodormancy (s-endodormancy) (Campoy et al., 2011, Faust et al., 1997, Heide, 2008). Deep and shallow endodormancy periods are distinguished by whether buds can respond to external dormancy breaking agents. Deciduous fruit trees need to be exposed to a minimum period of low temperature to overcome their dormant period and resume growth. The requirement of low temperatures that a plant needs to resume growth is referred as chilling requirement. Endodormancy ends with the accumulation of cold and fulfilling of the bud chilling requirement. Buds can respond to dormancy breaking agents only after a certain fraction of their chilling requirement has been received and transition from d-endodormancy to s-endodormancy took place. Endodormancy weakens during s-endodormancy stages and eventually switches to ecodormancy when chilling requirement is satisfied. Cold temperatures of early spring suppress plant growth during the ecodormancy stage. In this stage, growth resumes whenever favorable temperature is perceived (Campoy et al., 2011, Faust et al., 1997, Lang et al., 1987, Westwood, 1993).

Plant cold hardiness

According to Levitt (1980), cold hardiness represents the ability of plants to survive freezing temperatures in nature. The term stress resistance was introduced to meet the need of a quantitative terminology by scientists, and stands for the ability of a plant to survive unfavorable conditions and even grow in their presence. As previously discussed, freezing resistance in plants consists in their capacity to avoid lethal freezing events, i.e. ability of freezing avoidance, and to tolerate non-lethal freezing events, i.e. ability of freezing tolerance.

Cold hardiness of plants fluctuates through time. It begins to develop in fall, when plant growth cessation occurs, and it is lost rapidly in spring when growth resumes. The process of gaining hardiness is called acclimation and the process of losing hardiness deacclimation. Acclimation is induced by short-days and coincides with growth cessation. Deacclimation happens when plants overcome endodormancy and hardiness has been lost rapidly due to the fulfillment of chilling requirement and thereby bud break occurs (Proebsting, 1970, Weiser, 1970). For this reason, economic losses can occur due to untimely freezing temperatures before acclimation in the fall or after deacclimation in the spring (Ashworth and Wisniewski, 1991), especially in warmer regions. In Georgia, winters are generally warm and rarely induce cold injury in tissues. Contrary, in early spring in Georgia, tissues become deacclimated and cold injury can be a problem. Plant hardiness can be also variable between tissues, even after plants are fully acclimated. Hereafter, we will focus on the most critical and vulnerable tissue for fruit set and development - the reproductive tissue.

Supercooling is an important freezing avoidance mechanism utilized by peach plants. The ability of peach floral buds to supercool and survive freezing temperature has been previously confirmed by many researches (Ashworth and Wisniewski, 1991, Quamme, 1978, Quamme et al., 1995). In peach freezing experiments, ice inoculation, which would disrupt the supercooling status of flowers, proved to raise the lethal temperature that damaged 50% of excised flowers. This demonstrated that peach floral buds supercool during winter (Quamme et al., 1995). Water in the peach bud scales and bud axis freezes first when temperature drops with water inside of the bud remaining liquid due to supercooling. These two freezing events, water freezing in adjunct tissues

and supercooled water freezing inside the bud, occur distinctly in peach floral buds (Ashworth and Wisniewski, 1991). Similar phenomena were reported as "extraorgan freezing" by Sakai (1979) in conifers (Quamme, 1978, Sakai, 1979). It is proposed that isolating extracellular freezing from floral bud tissues reduces risk of freezing damage to the internal structures in the flower bud (Endoh et al., 2014).

Research has been done to explain the localization of ice crystals and the process of supercooling. Quamme et al. (1995) found a "barrier" that prevented ice propagation from the flower bud axis to the developing flower organs, which induced a deeper supercooling of the flower buds. The cells of the "barrier" area included provascular strands and a basal zone (BZ) at the flower base featuring thick cell walls and small cell volumes. Water is drawn to bud scales from these regions, therefore it forms a dry zone that impedes the progression of ice, facilitating the full expression of supercooling. In support of the theory above, previous research revealed the relationship between xylem continuity and the ability to supercool (Ashworth, 1984). In the floral buds of six *Prunus* species that supercool, the capacity to deep supercool stopped with the vascular tissue resuming differentiation after dormancy. *Prunus* species that do not supercool have xylem vessels that run the length of the raceme and vascular tissue, which enter the individual bud or floret during winter. These studies in *Prunus* suggested that the xylem acts as a conduit of ice propagation into the floral buds.

Aside from the separation from external ice, localized ice formation by intrinsic nucleating sites of plants ensures that water stays liquid in deep supercooling tissues. In peach, plant originated ice nuclei were found in stems that initiate ice formation outside floral bud at a warmer, subfreezing temperatures (Ashworth and Davis, 1984, Ashworth

et al., 1985, Gross et al., 1984, Gross et al., 1988). The function of peach intrinsic ice nuclei started to develop at midsummer and were completed by late August (Gross et al., 1988). In acclimated peach floral buds, localized ice formation sites were observed within bud scales and bud axis, but not inside of developing floral bud tissues (Ashworth et al., 1989, Quamme, 1978). After bud deacclimation, artificial freezing to -5° C produced large ice crystals within not only bud axis and scales, but also developing floral organs, indicating that the supercooling ability of floral buds was lost (Ashworth et al., 1989). Intrinsic ice nuclei stay active after acclimation, although no longer contributes to freezing resistance of peach flowers (Andrews et al., 1983, Ashworth and Davis, 1984, Gross et al., 1984). Similarly, Ishikawa and Sakai (1985) suggested that localized ice formation in bud scales rather than florets is induced by intrinsic ice nucleating sites in scale tissues of Chinese cornel dogwood (*Cornus officinalis* Siebold & Zucc.).

Even within the same buds, different structures show different susceptibility to cold. In the case of blueberry, many attempts had been made to explore differences of cold hardiness between floral organs. A study of highbush blueberry (*Vaccinium corymbosum* L.) examined cold susceptibility of different organelles within open flowers with lethal temperature $_{50}$ (LT_{50}) being calculated for each one of them (Rowland et al., 2013). It was reported that the flower organs were classified from the most sensitive to the hardiest as follows: corolla, filament, anther, style, exterior ovary, stigma, ovules, interior ovary, and placenta (Rowland et al., 2013). Previous freezing studies provided similar susceptibility of floral structures and inspected the critical structure for fruit set and eventual yield as well (Gupton, 1983, NeSmith et al., 1999). Freezing damage of five rabbiteye blueberry cultivars (*Vaccinium virgatum* Reade) floral buds were studied after a

mild natural freeze (Gupton, 1983). This study showed no significant difference of fruit set between hand-pollinated and open-pollinated frost-damaged flowers (Gupton, 1983). Therefore, Gupton (1983) concluded that reduced fruit set is a direct consequence of pistil damage but not of loss of pollinators. In another study of rabbiteye blueberries, 'Brightwell' flowers exposed to -1 °C showed a sharp decline of pollination by bees without presenting visual damage to structures associated with pollination such as corollas, styles, and ovaries (NeSmith et al., 1999). NeSmith et al. (1999) hypothesized that some 'hidden damage' beyond visual inspection was the cause of the potential freezing damage to floral buds.

In peach floral buds, cold hardiness of different parts were studied after treated with freezing temperatures under laboratory conditions (Quamme 1974). Flower bud parts were listed from the most susceptible to the most resistant to freezing temperatures as follow: pistil, anthers, corolla, calyx, and pedicel (Quamme 1974). Yet some other observations suggested that there was no definite order of cold hardiness to tenderness among peach floral bud structures (Oberle, 1957). In this context, some field observations of a peach orchard in Virginia were made after a natural freeze. Some flowers opened without pistils, since the pistils were frozen and aborted, while other structures developed normally. Other flowers showed exactly the opposite with pistils healthy while sepals, petals and stamens, were damaged by freezing and stopped growing (Oberle, 1957).

The differences between blueberry and peaches floral buds freeze damage could be attributed to the observation that blueberry floral buds did not supercool under a natural cooling rate of 1-2 $°C \cdot h^{-1}$, while dormant whole peach flower buds exhibited supercooling under a cooling rate of 2 $°C \cdot h^{-1}$ (Ashworth, 1982). This variation may

contribute to the differences in freezing damage of the floral buds. However, information about floral structure cold hardiness and critical structure for fruit set and yield are rare.

Stems also undergo the acclimation and deacclimation process. In freezing experiments using acclimated stems of peach, two freezing events can be detected as well. The first freezing event is associated with water freezing within the non-living xylem vessels. This event does not correlate with tissue damage. The second freezing event occurred at lower temperature and it was associated with damaged xylem ray parenchyma cells. These results suggested that water in stem tissues supercool (Burke et al., 1976).

Measuring cold hardiness

The ability of peach floral buds to tolerate freezing can be measured by artificial freezing test (Aslamarz et al., 2010). This test involves applying an artificial freezing treatment to the tissue and examining it for freezing injury. In this test, a programmable freezing chamber or freezing bath is usually utilized to apply artificial freezing temperatures to test samples (Rowland et al., 2013). Samples are held at a constant temperature just below 0 °C to reach a uniform temperature distribution within the chamber and tissues. This step also allows extracellular water to freeze and allows cells to reach an equilibrium status to avoid freezing of supercooling water inside of cells. After equilibrium has been achieved, the temperature within the chamber is decreased at a standardized cooling rate. A series of treatment temperatures are assigned to samples. At each designated temperature, a group of samples is removed from the freezing chamber and transferred to room temperature to thaw and to recover. Samples are

incubated at above-zero-temperatures to allow oxidation of freezing-injured cells, which will develop discoloration serving as a visual indicator of freezing damage (Westwood, 1993). Each sample will be visually examined for discoloration and rated for damage. Once freezing injury damage has been rated, the freezing temperature that causes damage to 50% of the bud samples can be calculated. This temperature is known as LT_{50}, which stands for 50% lethal temperature (Bigras and Colombo, 2013, Levitt, 1980, Stergios and Howell, 1973).

Artificial freezing test is a standard test that estimates the level of cold tolerance of plant tissues and usually offer a good estimator of field survival (Levitt, 1980). However, field conditions cannot be simulated by test conditions, and even field conditions are inconsistent from time to time. Also, the evaluation for injury is not objective but subjective to the evaluator's personal perception of color and is not accurate with small samples (Burr et al., 1990).

Another aspect of freezing resistance is freezing avoidance, which allows water inside of plants cells remain liquid even below freezing temperatures. Differential thermal analysis (DTA) is a useful technique to demonstrate freezing events inside of tissues and, therefore, to assess the ability of tissue's freezing avoidance. Thermal-electric modules (TEM) are used for DTA analysis to perceive the temperature difference between samples and the inert reference materials, and convert the signal into voltage along the process of the artificial low temperature treatment. When water freezes in plant tissues, the transition from liquid to solid phase, in other words, a freezing event, releases energy changing the sample temperature which can be detected by TEMs (Quamme et al., 1972). The properties of DTA make it a convenient and objective test to measure the

freezing avoidance ability of a sample, with results available within few hours after sample collection.

Two possible freezing events can be detected in tissues that are supercooled. High temperature exotherm (HTE), usually occurs few degrees below 0 °C, represents freezing of extracellular water and usually is non-lethal. Low temperature exotherm (LTE), which happens at a much lower temperature, is associated with freezing of supercooled water that ruptures cell membranes and causes lethal damage (Ashworth, 1982, Biermann et al., 1979, Burke et al., 1976, George et al., 1974b, Ishikawa and Sakai, 1985, Levitt, 1980, Wolf and Pool, 1987). In peach, both HTE and LTE can be detected in cold acclimated floral buds and stems (Ashworth, 1982, Ashworth et al., 1983).

Although DTA measures only the temperature of water freezing in samples and not freezing resistance, good relationships between LTE from DTA and LT_{50} or LST (least survival temperature) determined by artificial freezing tests have been previously reported (Quamme et al., 1972, Quamme et al., 1973). Quamme et al. (1972) examined apple stems from October to April. They noted that temperature of LTE was closely associated with the LST from artificial freezing test (lowest temperature where plant tissues didn't exhibit injury) (Quamme et al., 1972, Quamme et al., 1973). More studies revealed close relationships between the mean exotherm temperature and LT_{50} using artificial freezing tests of dormant flower buds among *Prunus* spp. and grapes (*Vitis vinifera* L.) (Andrews and Proebsting Jr, 1987, Proebsting Jr and Sakai, 1979, Quamme, 1974, 1983, 1986, Quamme et al., 1975). Although Wolf and Pool (1987) found that the median LTE determined by DTA was about 1.5 °C to 2 °C higher than the LT_{50} determined from artificial freezing test of dormant grape floral buds. They suggested that

the 2 °C temperature difference was due to a lag between measured air temperature adjunct to the bud and the actual freezing temperature of the bud tissue, and therefore DTA had the potential to offer an accurate estimation of hardiness. Similarly, in blueberry, LTE determined by DTA of excised buds correlated well with LST_{66} from artificial freezing test (lowest temperature at which at least 2 out of 3 buds survived for each stem position) (Biermann et al., 1979).

The DTA analyses and artificial freezing tests, nevertheless, are affected by the experimental conditions such as cooling rate and sample types. For example, water status and cooling rate affected the DTA profiles of blueberry floral buds (Biermann et al., 1979, Flinn and Ashworth, 1994) and the artificial freezing test results of non-natural supercooling Southern Magnolia leaf parts (*Magnolia grandiflora* L.) (Lindstrom and Dirr, 1991). Similarly, bud excision, cooling scheme, preconditioning temperature, and moisture loss in controlled conditions were shown to influence DTA for grape buds (Kovács et al., 2002, Quamme, 1986, Wolf and Pool, 1987). The factors influencing artificial methods to determine plant cold hardiness, especially in peach, will be further illustrated below.

Factors that affect cold hardiness measurement

1. Environmental conditions

a. Water Content

Water content in buds fluctuates throughout the different dormancy stages (Quamme, 1983). These changes have been associated with cold hardiness (Hewett et al., 1978). In endodormancy, free water in buds is at its lowest level and more water is in

bound status. In peach, other conditions that enhance dormancy such as low temperature and short-days also increase bound water level in buds (Yooyongwech et al., 2008). Faust et al. (1995) observed that in vegetative buds of apple during endodormancy, approximately 30% of the bud's water is in bound state before its chilling requirement was met. Free water rose to around 81% after the chilling requirement was completely fulfilled. Water rapidly converts to a free state when growth is resumed.

Bud water content and its effect in cold hardiness was investigated in several species. Kader and Proebsting (1992) reported that dehydrated buds can supercool to lower temperatures compared to hydrated buds of *Prunus serotina* Ehrh. and *Prunus padus* L. (Kader and Proebsting, 1992). In peach floral buds, it has been found that seasonal changes in the supercooling point were significantly correlated with seasonal changes in water content of whole buds, floral primordia, vascular tissues (Quamme, 1983) and specifically, water content of pistil during deacclimation (Durner and Gianfagna, 1991). Aslamarz et al. (2010) suggested that buds begin to absorb water after its chilling requirement is fulfilled, thus giving rise to more ice nucleation sites and decreasing buds' ability to supercool. This correlation between increased water content and loss of cold hardiness also holds true for Chinese cornel dogwood (Ishikawa and Sakai, 1985), sweet cherry (Andrews and Proebsting Jr, 1987), blueberry (Biermann et al., 1979, Bittenbender and Howell, 1975), and grape (Kovács et al., 2002).

Aside from water content changes within a bud due to physiological processes occurring in nature, moisture loss during sample preparation can also influence cold hardiness determination. Kovács et al. (2002) investigated moisture loss of excised grape buds. Approximately 10.5% and 6.9% water content losses were observed on excised

‘Vignoles’ and ‘Norton’ grape buds, respectively, after being exposed to open air in room temperature for 5 min. In addition, they tested the effect of dehydration on LTE, and found that lower LTE temperatures (P<0.001) were sufficiently produced by only 6.5% and 4.3% water loss of ‘Vignoles’ and ‘Norton’ buds, respectively. Moisture changes during test manipulation were found to confound DTA test results.

b. Agricultural Practices

Many agricultural practices have been shown to affect critical bud freezing temperature. Thinning (Byers and Marini, 1994), irrigation (Hewett et al., 1978, Layne et al., 1994), fertilization (Pellett and Carter, 1981), and rootstock selection (Durner, 1990b, Layne and Ward, 1978) have an impact on bud cold hardiness.

i. Rootstock

Many studies in peaches have focused on the use of different species as rootstocks to improve peach cold hardiness. In rootstock trials conducted in Missouri with ‘Redhaven’ scions, no significant differences in critical bud temperature estimated by artificial freezing test of flower buds were detected among eight peach rootstocks in mid-winter. In early spring, on the other hand, ‘Redhaven’ grafted on ‘Lovell’ trees had hardier floral buds than of those grafted on other rootstocks, followed by grafted trees on ‘Damas 1869’. The least hardy buds were identified on ‘GF 677’, ‘Halford’, and own-rooted ‘Redhaven’ trees (Warmund and Slater, 1988). A similar study was performed in Missouri, Ohio, and South Carolina (Warmund et al., 2002). In this study, it was reported the presence of differences of floral bud cold hardiness in mid-winter. In January 1997,

rootstocks differences in cold hardiness were significant. ‘Ishtara’ and ‘Bailey’ rootstocks produced the cold hardier ‘Redhaven’ buds whereas ‘Montclar’ and ‘BY520-8’ produced the most tender. Cold hardiness of flower buds from grafted trees on ‘Lovell’ and all other rootstocks besides the ones described above were in the middle for their level of hardiness and were very close to each other (Warmund et al., 2002).

In a field study, the effect of rootstocks on flower bud cold hardiness of the peach cultivar ‘Redhaven’ was evaluated after a natural freeze in 1987, with air temperature dropping to -26 °C on February 15. The percentage of flower bud survival was estimated after the freeze. ‘Redhaven’ trees grafted on ‘Citation’ and ‘Damas’ had the highest bud survival. The lowest bud survival were reported for trees grafted on ‘Amandier’ and ‘GF655-2’ (Brown and Cummins, 1988).

ii. Thinning

Byers and Marini (1994) reported a negative relationship between crop load of previous season and the percentage of buds alive after a natural freeze early at bud swell in the current season. Also, they showed that bloom thinning and fruit thinning at moderate crop densities improved cold hardiness of peach flower buds in late winter (Byers and Marini, 1994). Similarly, pruning during dormancy was shown to have a negative effect on bud cold hardiness by decreasing the ability of the buds to re-harden after a period of warm temperature during winter (Durner, 1990a). In the same context, Rodrigo (2000) hypothesized that the thinning practices may influence cold hardiness by altering crop load and nutrition state of fruit trees.

iii. Fertilization

Nitrogen fertilization was reported to improve cold hardiness of peach flower buds (Chandler, 1913, Cullinan, 1931, Proebsting, 1961). Fertilization rates from 0 to 0.92 kg per tree had a positive relationship with increase bud survival rates from mid-December until mid-March in the following winter (Proebsting, 1961). Similar correlation between N fertilization and improved cold hardiness was also described by Chandler (1913) and Cullinan (1931).

On the other hand, some researchers have found no relationship between N fertilization and cold hardiness (Byers and Marini, 1994, Cooper and Wiggins, 1929, Edgerton and Harris, 1950). A study in Georgia showed that although N fertilization had no effect on cold hardiness of flower buds of peach cultivar 'Elberta', it did however contribute to wood hardiness (Higgins et al., 1943). In other studies, a negative correlation between N fertilization and cold hardiness was also observed (Crane, 1924). Green and Ballou (1904) suggested that high N fertilization rates may cause over-fertilizing, promote rapid growth and weak woods. Peach trees over fertilized with N were more susceptible to low temperature injury.

iv. Irrigation

Under trickle irrigation, flower buds were hardier in non- irrigated trees versus irrigated trees. The authors hypothesized that full-season irrigation promoted growth and thus delayed acclimation in fall. However, the bud hardiness was negligible (Layne et al., 1994).

Overhead irrigation has been utilized as a frost protection practice. A constant supply of water maintains the temperature of plants around 0 °C by releasing heat when the water freezes (Westwood, 1993). This method reduces the damage caused by a freeze when conducive conditions for freeze protection are available.

2. Test Protocol Factors

a. Cooling rate

It has been found that the ability of DTA to detect LTE is affected by the cooling rate. In the case of blueberry flower buds, a cooling rate of 2 ˚C·h^{-1} was not able to detect LTEs in any attached flower buds, while the cooling rate of 10 ˚C·h^{-1} allowed several LTEs per buds to be identified (Flinn and Ashworth, 1994). It can be hypothesized that, in this study, the greater cooling rate did not allow enough time for water to migrate out of the flower bud. At a certain temperature, more water was still trapped inside of the bud under a fast cooling rate than the slow cooling rate. As discussed before, low water content facilitates freezing avoidance with the water inside of the tissue being supercooled and freezing at a lower temperature. When the cooling rate is sufficiently slow, the equilibrium status can be achieved, no deep supercooling would happen, and no LTEs could be detected.

Further evidence supporting the theory of the cooling rate affecting the DTA profile by changing water behavior were reported in many other species. Excised dormant floral buds of azalea were treated with different cooling rates: 8.5 ˚C·h^{-1}, 18.8 ˚C·h^{-1} and 37 ˚C·h^{-1}. Only one LTE at elevated cooling rates (18.8 ˚C·h^{-1} and 37 ˚C·h^{-1}) was detected from one bud containing several primordia, indicating these primordia froze

at the same temperature. The lower cooling rate (8.5 ˚C·h^{-1}) resulted in many LTE peaks at lower temperatures in the DTA profile (George et al., 1974b). In this case, the slow cooling rate ensured adequate dehydration disrupting the continuity of water between several primordia and blocking the path for ice to invade separate primordia. Therefore, primordia in the same buds froze separately under the slow cooling rate. In another study, the median LTE temperature of the conifer primordial shoot of winter bud was lower under a slow cooling rate (3 ˚C·h^{-1}) compared to a faster cooling rate (6.6 ˚C·h^{-1}) due to reduced water content (Sakai, 1982). Low temperature exotherms with the slow cooling rate were observed as smaller peaks within the DTA profile, suggesting water may migrated out of buds at this point. At the slowest cooling rate of 5 °C per day, no LTEs were observed in the conifer primordial shoots, implying that at this cooling rate the equilibrium condition was reached (Sakai, 1982).

In the study of peach flower buds, Ashworth (1982) noted that the cooling rate at the beginning of the freezing process largely affected LTEs detection. Excised whole buds were tested with three cooling schemes, 2 ˚C·h^{-1}, 20 ˚C·h^{-1}, and 2 ˚C·h^{-1} (down to -10 °C) then 20 ˚C·h^{-1}. Only at the cooling rate of 20 ˚C·h^{-1} no LTEs were observed for the flower buds. It was concluded that the cooling rate at the beginning of the freezing process is the most important. At the beginning of the freezing process, buds have not dehydrated and the bulk of freezable free water was still inside of flower buds. Slow cooling rate at the beginning allowed time for the bulk water to redistribute, and created a preferential condition for deep supercooling and detection of LTEs (Ashworth, 1982). Flinn and Ashworth (1994) suggested that to reflect the real cold hardiness of plant tissues in the field, a cooling rate closely resembling nature should be chosen for DTA,

i.e., 1 to 2 ˚C·h^{-1}. This cooling rate, however, may not always necessarily be the most workable one.

In an earlier study, Biermann et al. (1979) examined a wider range of cooling rates in highbush blueberry and concluded that the most workable cooling rate for test was 8 ˚C·h^{-1}, close to the cooling rate determined by Flinn and Ashworth (1994) that yielded several LTEs. Furthermore, when examining higher cooling rates, Biermann et al. (1979) noted that 65 ˚C·h^{-1} and 33 ˚C·h^{-1} resulted in overlapping exotherms when LTE happened at higher temperatures. Similarly, in the study of Flinn and Ashworth (1994), detached buds showed higher temperature of LTE with higher cooling rates. There are exceptions, however, when LTE temperature estimations are independent from cooling rate. In the case of grape, Quamme (1986) examined two cultivars and discovered that, for attached dormant grape buds, cooling rate within the range of 1.5 ˚C·h^{-1} to 40 ˚C·h^{-1} did not significantly affect the estimation of LTE temperatures. A later study recorded a difference between LTE temperatures of attached grape flower buds under two cooling rates of 2 ˚C·h^{-1} and 5.6 ˚C·h^{-1}, although significant, but only for 1 ˚C (Wolf and Pool, 1987).

In the case of stems, a study of apple stems during winter concluded that the LTE temperature was unaffected by cooling rate (Quamme et al., 1972). Since stem, as opposite of flower buds, serves as an ice sink, a low cooling rate does not change water behavior in the stems as it does to flower buds.

b. Sample size

Sample size can also affect freezing resistance estimation using the artificial freezing test and DTA. In a study with pure water, a logarithm relationship between water drop diameter and mean freezing temperature was recognized (Ashworth, 1991). Likewise, a logarithmic relationship between sample fresh weight of stem and nucleation temperature was also noted in both tomato and peach plants (Anderson and Ashworth, 1985, Ashworth and Davis, 1984, Ashworth et al., 1985). Smaller samples tend to supercool to lower temperature than larger samples and whole plants. Anderson and Ashworth (1985) monitored freezing temperatures of many tomato tissues, including leaf disk, petiole sections, small seedlings and stem sections. They found that, although the sample freezing temperature decreased to lower temperatures with smaller sample sizes, sample size had limited effect on nucleation temperature of ice nucleation-active (INA) inoculated plants, ranging from -3 to -2 °C with a sample weight from 0.4 g to 180 g. For not inoculated tissues, stem sections of 1 g could supercool to -6 °C, while plant size of 100 g supercooled only to -2 °C (Anderson and Ashworth, 1985). For peach stems, when fresh weight was larger than 5 g, nucleation temperature reminded constant about -2.5 °C (Ashworth and Davis, 1984). Considering these results, they further suggested that small samples tend to overestimate the depth of supercooling thus overestimating the cold hardiness of plants (Anderson and Ashworth, 1985, Ashworth and Davis, 1984). The same relation was also demonstrated in stem samples of Eucalyptus species (Scarascia-Mugnozza et al., 1989).

c. Excision

Sample excision has a direct effect on ice nucleation temperature as well. Excision not only reduces sample size as discussed above, but most importantly, changes morphological characteristics of plant samples. Flinn and Ashworth (1994) found a correlation between LT_{50} of blueberry flower buds and stem excision. LT_{50} of excised blueberry flower buds was higher than that of attached buds except for one collection date in their test. The greatest difference between LT_{50} of buds with and without stem reached 14.5 ˚C. They therefore concluded that excised buds tended to yield more conservative results when estimating cold hardiness by artificial freezing test. Another study comparing artificial freezing test of blueberry floral buds using detached shoot and whole plant found no significant difference between these two methods (Rowland et al., 2013).

DTA profiles have been shown to be affected by sample excision. Median temperature of HTE of excised buds was higher than that of attached buds in blueberry (Flinn and Ashworth, 1994). Similarly, LTE was only observed under a fast cooling rate of 10 ˚C·h^{-1} in attached buds but not under a slow cooling rate of 2 ˚C·h^{-1}. In contrast, excised buds could produce LTE with both fast and slow cooling rates in general. Under a fast cooling rate, median LTE temperatures of attached buds were lower than those of excised buds except for one sample date (Flinn and Ashworth, 1994). Stem excision yielded higher temperature LTEs in flower buds of grape and peach in comparison with un-excised samples (Quamme, 1986).

On the other hand, excised flower buds of both peach and blueberry showed similar nucleation temperatures as non-inoculated attached buds when inoculated with

INA bacteria. This study supported the role of stems as ice nucleating activators and ice sinks that would decrease ice nucleation temperature of flower buds when attached to stem by drawing water out of floral bud (Flinn and Ashworth, 1994, Gross et al., 1984).

Ashworth (1982) studied the relationship of supercooling and viability of different tissues of peach to confirm the importance of structural differences associated with supercooling. LTEs were still observed when peach flower buds attached to stem were kept structurally intact and pretreated with a low temperature of -25 °C that killed only the developing bud tissues, but not the bud axis. Supercooling was prevented and no LTE occurred only when both bud axis and floral bud tissues were killed by a low temperature of -40 °C (Ashworth, 1982). These results confirmed the important role of stems as ice sinks that affect water behavior during artificial freezing test and DTA test.

The effect of primordia excision on DTA was also investigated in many species. In the case of azalea, Graham (1971) demonstrated that the freezing temperature of excised primordia is independent of the cooling rate, although DTA profiles of whole buds largely changed under different cooling rate (George et al., 1974b). Exotherm temperature of azalea primodia under cooling rates of 3 $^{\circ}C \cdot h^{-1}$ and 180 $^{\circ}C \cdot h^{-1}$ were close (Graham, 1971). Similar phenomenon was observed in peach. As previously described, identification of LTE for whole peach flower buds was affected by the cooling rate of the freezing experiments, while isolated primordia of peach flower buds produced LTE regardless of cooling rate (Ashworth, 1982). These results together implied that the intact structure, in specific stem attachment, is critical for expression of supercooling, thus greatly affecting the DTA estimation.

d. Preconditioning treatment

Researchers have demonstrated that sample storage and preparation temperature can also alter the result of cold hardiness estimations. Ashworth (1982) found that low temperature treatments can lead to acclimation of samples and thus can be used to amplify differences between cultivars before cold hardiness determination. Among three grape cultivars that were tested, buds were subjected to -5 °C for 7 days followed by -10 °C for 3 days. Cold hardiness was improved significantly before chilling requirement was met, but not after. A decrease of LTE temperature was observed in both cases (Ashworth, 1982).

A similar effect in LTE was also noted on peach flower buds (Quamme, 1983). After a 10-day conditioning period of 0 °C and -10 °C, LT_{50} of flower buds of the 0 °C treatment increased overall, indicating acclimation, while LT_{50} of -10 °C treatment decreased, indicating deacclimation. Water movement was also noted. Under the -10 °C treatment, water content of flower primordium decreased when water content of whole bud remained the same, indicating water migrated from primordium to other flower bud parts. The dehydration, similar to acclimation process, may have contributed to the increased cold hardiness estimation by the freezing test (Quamme, 1983).

On the other hand, warm temperatures induced deacclimation of samples. The effect of warm temperature, moisture content, and interaction between them on cold hardiness determination were studied for highbush blueberry flower buds (Bittenbender and Howell, 1975). After the plant chilling requirement was fulfilled, flower buds of blueberry appeared to be 3 °C less hardy after exposed to 25 °C for 24 h than buds stored at 2 °C (Bittenbender and Howell, 1975). Similar de-hardening effect by high

temperature were reported for peach and blueberry flowers (Proebsting and Mills, 1972). High temperatures can also cause loss of cold hardiness in peach as early as mid-winter after chilling requirement has been partially fulfilled (Proebsting and Mills, 1972). Altogether, these reports suggested storage and pre-test temperature can play a very influential role during cold hardiness estimation.

Summary

In this review, we discussed the principles of how plants survive sub-freezing temperatures, especially perennial fruit crops. In addition, the standard methodology used to determine cold hardiness was summarized. Year after year seasonal changes of weather are occurring. It is becoming critical to address cold hardiness in fruit crops in a fast and accurate way. DTA serves as a promising solution for its convenience and quick availability. To use DTA as a practical method in real world fruit production to evaluate cold hardiness in the field, its creditability must first be established and compared to a standard method such as artificial freezing test. These two possible approaches could be used to bridge the gap between laboratory test and field observations.

As we described before, DTA estimations are affected by various experimental conditions and tend to deviate from field observations. DTA does not directly measure cold resistance, but the temperature of nucleation, which is a physiological parameter that is related with cold resistance. The information from DTA may be able to be translated to accurate cold hardiness data obtained from artificial freezing test using different statistical methods. Large sample size and multi-year DTA data will be required to reduce

variability and to improve calibrations year-after-year against standard tests (Burr et al., 1990).

Alternatively, a better understanding of the relationship between ice nucleation temperature and cold damage can help revealing a specific meaning of the DTA profile with respect to the different LTEs. As previously described, HTEs in floral buds reflect water freezing in bud axis and scale, which is not directly linked to damage. However, dehydration stress is imposed by ice in those regions after HTE appears. Bud damage, therefore, can occur at temperature between HTE and LTE. It is important, in this case, to further investigate the cause of cold damage (dehydration or intracellular ice) in specific tissues, and how these tissues are critical to fruit set and yield. Through the understanding of the nature of HTEs and LTEs, DTA can be improved for precision and accuracy by removing any factors that could affect DTA results. This will further support the use of this technique as a powerful tool for growers to determine the level of cold hardiness and critical bud freezing temperature of their crops.

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CHAPTER 2

ARTIFICIAL FREEZING TEST AND CRITICAL BUD FREEZING TEMPERATURE DETERMINATION OF PEACH IN GEORGIA[1]

[1]Liu, J. et al. To be submitted to *HortScience*.

Abstract

Freezing damage is a major cause of economic losses in peach production in the Southeast U.S. Gaining knowledge about peach plant cold hardiness can help forecasting the probability of freezing damage and economic losses that could be produced due to upcoming freezes. It will aid researches and growers to optimize frost protection strategies to avoid such losses. The standard test of evaluating cold hardiness of plant tissues is the artificial freezing test. This test examines the cold damage of plant tissues directly after an artificial low temperature treatment. Cold hardiness measured by the artificial freezing test is known as critical bud freezing temperature and can be expressed as lethal temperature that caused damage to 50% of samples (LT_{50}). Certain test conditions, such as sampling type, can affect the artificial freezing test. Also, cold hardiness information of peach floral buds at different stages grown in the Southeastern U.S. are not yet measured and unavailable to growers. The objectives of this study are to explore the effect of sampling types on critical bud freezing temperature determined by artificial freezing test and to develop cold hardiness reference values of peach for Georgia growers. Artificial freezing tests were conducted with two cultivars, 'Elberta' and 'Flavorich', and three different sampling types (tissues on 5 cm stems, tissues on 20 cm stems, and excised floral and vegetative buds) throughout three consecutive winters. Significant LT_{50} differences were reported among collection dates, cultivars, sampling types, and tissue types (floral bud, vegetative bud, and stems) ($P<0.0001$). Floral buds tended to be the most cold susceptible tissues during and after deacclimation for both cultivars. This stage is when most yield loss occurs due to cold damage in Georgia. Floral buds attached to stem no shorter than 5 cm were determined to be optimal for artificial

tests, and were recommended for artificial freezing test. Critical bud freezing temperature of peach floral buds from ‘Elberta’ and ‘Flavorich’ cultivars were recorded through October to March each season. Floral buds of ‘Elberta’ tended to be hardier than 'Flavorich' buds. These buds also deacclimated later than 'Flavorich' buds. Our study optimized the protocol used for the artificial freezing tests for our conditions. In addition, this study yielded the critical bud freezing temperature estimations for an early season variety ‘Flavorich’ and a mid-season variety ‘Elberta’ which represent Georgia peach production. This information can facilitate frost protection management for Georgia peach growers.

Introduction

Peach [*Prunus persica* (L.) Batsch] is an important fruit crop and the state fruit of the state of Georgia. Georgia produced 40,600 tons of peach in 2015 with a farm gate value of 49 million dollars (USDA, 2016; Wolfe and Stubbs, 2016). A major cause of yield losses in peach production are freeze events (Aslamarz et al., 2010). The process of cold hardening plus a series of physiological changes in plants are called acclimation and the loss of cold hardiness is deacclimation (Proebsting, 1970, Weiser, 1970). In the Southeast U.S., low temperatures during winter do not normally cause damage to peach floral buds, however, peach floral buds are especially susceptible to freezing damage after deacclimation. In nature, peach floral buds start to develop cold hardiness from late summer or fall in preparation of winter's cold temperatures to achieve the greatest hardiness and deepest dormancy (Faust et al., 1997, Westwood, 1993). Floral buds will break when sufficient chilling accumulation is achieved followed with warm weather in late winter and early spring (Faust et al., 1997, Westwood, 1993). Economic losses can occur due to both untimely freezing temperatures before acclimation in the fall or after deacclimation in the spring (Ashworth and Wisniewski, 1991). It is important to assess the cold hardiness level of plants prior to a freeze event to estimate possible damage and loss of floral buds and to provide information to growers in aid of making the best frost protection strategies to mitigate losses of yield.

To monitor cold hardiness of peach floral buds, artificial freezing tests can be used. It is a standard test and usually offer a good estimator of field survival (Levitt, 1980). This test involves applying controlled freezing temperatures to whole plants or plant tissues, and evaluating bud survival or damage caused by low temperature

(Aslamarz et al., 2010). Damaged tissues usually are oxidized and develop discoloration of brown or black, and thus makes visual measurement of low temperature damage possible (Proebsting Jr and Sakai, 1979). From the evaluation, critical bud freezing temperature (critical bud temperature in short in following context) can be calculated as LT_{50}, lethal freezing temperature that killed 50% of all samples. The methodology of the artificial freezing test must be standardized to ensure that results are comparable among different times, locations, and materials (Proebsting Jr and Sakai, 1979). Test conditions are also desired to mimic as close as possible natural freezing events and to provide accurate estimation of cold hardiness of a crop in field conditions (Flinn and Ashworth, 1994).

It is known that intact anatomical and morphological features of peach buds facilitate supercooling (Quamme et al. 1995). Also, sample mass largely affects nucleation behavior in peach tissues (Ashworth et al., 1985). The study of how these features would affect the artificial freezing tests are rare. Performing artificial freezing test with excised buds is convenient, however, logically, buds on longer stems would more closely simulate field conditions. Thus, one of objectives of this study is to explore how different sampling types affect critical bud temperature assessments by artificial freezing test. In addition, the critical bud freezing temperatures of 'Elberta' peach had been previously reported using artificial freezing tests by researchers of Washington State University (Ballard et al., 1999). Yet, no data of critical bud temperature for peach is currently available for the Southeastern U.S. region. Thus, it is necessary to determine the critical bud temperature of peach floral buds for the benefit of peach growers in the Southeast U.S.

Materials and Methods

Plant material

Twigs were randomly selected from peach cultivars 'Elberta' and 'Flavorich' trees grafted on 'Guardian' rootstock planted in 2010 in a commercial orchard at Fort Valley, GA. These commercial orchards were managed following recommended guidelines in the Southeastern peach, nectarine, and plum pest management and culture guide (Horton et al., 2010). Sample collection started on October and ended at end of March of the following year for three consecutive seasons starting in 2015 until 2017. Sample intervals varied from one sampling time per month in early fall to once a week in early winter until spring.

A total of 48 sampling dates were done for 'Elberta' and 43 samplings were done for 'Flavorich' in three seasons. For each sampling date, approximately 60 stems were collected. Samples were transported to the University of Georgia Griffin Campus, Griffin, GA in a cooler with ice. Samples then were kept in a refrigerator at 4 °C for no longer than a day before processing.

Stems were then carefully defoliated if leaves were still attached to the stems. Samples were prepared and separated into four different types: 5 cm stems with buds, 20 cm stems with buds, excised floral buds, and excised vegetative buds. Stems were cut into desired lengths and wrapped in a damp tissue paper at the basal end to prevent wilting. Similarly, excised buds were wrapped in a damp tissue paper.

Freezing and Thaw

A total of ten temperature treatments were used for analyses: 4 °C (control), -3 °C, -6 °C, -9 °C, -12 °C, -15 °C, -18 °C, -21 °C, -24 °C and -27 °C. Four reps of each sample type per cultivar per temperature treatment were prepared and sealed in a plastic bag, with each plastic bag representing a temperature treatment. Bags containing samples were hanged on metal rods and set in the freezing chamber (Temperature and humidity chamber PR-3FPH, Tabai ESPEC, Japan). Three temperature probes were installed to measure air temperature within the freezing chamber at different locations. Control samples were left in the refrigerator at 4 °C. The freezing chamber was set to a constant -2 °C overnight. Since in nature air temperature usually decreases 1-2 $°C \cdot h^{-1}$, cooling rates exceeding 1-2 $°C \cdot h^{-1}$ would cause rapid supercooling thus excessive freezing damage that is generally missing in field conditions. Setting samples overnight at -2 °C would prevent supercooling and allow them to reach a uniform distribution of temperature. Freezing tests were started by decreasing the temperature in the freezing chamber at a rate of 4 $°C \cdot h^{-1}$ from -2 °C until it reached -27 °C. Each bag was rapidly removed from the freezing chamber when the temperature inside the chamber reached their assigned temperature treatment. After removal, bags were placed in a refrigerator at 4°C to thaw.

Visual Evaluation

Samples were kept in a refrigerator for a week before they were dissected and visually rated for damage. Flower buds and vegetative buds were excised from 5 cm stem and 20 cm stem for evaluation of the freezing damage of each temperature treatment. Buds were cut longitudinally with a razor blade and visually inspected for brown

discoloration under the stereoscope. The developing flower bud with browning color were scored as dead, while buds that remained green and without any discoloration were scored as alive. For stems, a small piece of each stem was sliced off longitudinally with a razor blade to evaluate the discoloration of the cambium and the phloem. Stems with yellow and brown colored cambium and phloem were rated as dead, while stems that remained green were considered alive and free from injury. For each temperature treatment, eight floral buds and four vegetative buds from 5 cm stems, eight floral buds and four vegetative buds from 20 cm stems, and four excised floral buds and four excised vegetative buds were evaluated for each cultivar and temperature treatment. The number of dead or alive buds or stems of each temperature treatments were recorded.

Data Analysis

Data analyses were carried out using JMP Pro 13 software (SAS Institute Inc., Cary, NC, USA). A nominal logistic model was fitted to determine LT_{50}, i.e. the temperature at which 50% of buds or of stems were killed by freezing temperatures. Data was then analyzed using Student's *t*-test (α=0.05) to compare LT_{50} values between different treatments per sample date.

Results

Overall, all factors, including collection date, cultivar, sampling type (5 cm stems, 20 cm stems, and excised buds), and tissue (floral, vegetative, and stems), were all found to be significantly different for LT_{50} as determined by the artificial freezing test (P<0.001).

Effect of sampling types on artificial freezing test

For 'Elberta' in the first two seasons, floral buds of all three sampling types showed similar acclimation and deacclimation pattern. (Fig. 2.1A and 2.1B or Table 2.1). Floral buds LT_{50} for all three sampling types decreased from October, and plateau around December 1 in the first season and January 12 in the second season. Excised floral buds did not exhibit LT_{50} values as low as attached buds to stems. LT_{50} values began to increase around February 16 of the first season and February 23 in the second season and tended to fluctuate until the end of the sampling dates.

From December 2, 2014 to February 2, 2015 and January 12, 2016 to February 15, 2016 when LT_{50} values were lowest and relatively stable, excised floral buds of 'Elberta' tended to be less cold resistant than floral buds attached on 8 cm and 20 cm stems. This suggested that excision seemed to prevent fully expression of cold hardiness of floral buds (Fig. 2.1A and 2.1B or Table 2.1). Similarly, 'Flavorich' excised buds tended to be more cold susceptible than buds attached on stems before deacclimation. Differences between LT_{50} of excised buds and attached buds were small after deacclimation started (Fig. 2.1D and 2.1E or Table 2.1).

The third season was featured with a rare warm winter. Floral buds LT_{50} for both cultivars reached the plateau stage in the middle of December (December 12, 2016), which was early comparing to two previous years. For floral buds of both 'Elberta' and 'Flavorich', excised buds were rated as the least cold hardy across all sampling types especially before being fully acclimated when differences between LT_{50} of excised buds and attached buds being the largest (Fig 2.1C and 2.1F). Around January 11, 2017, when floral buds were completely acclimated, differences of LT_{50} between excised floral buds

and attached floral buds were small and continued until the end of the evaluation season in March (Fig 2.1C and 2.1F).

When comparing LT_{50} values estimated using the artificial freezing tests for floral buds on 5 cm stems and buds on 20 stems, only two out of 48 sampling dates (November 10, 2014 and October 27, 2015) reported significant differences among sampling types for 'Elberta' (Table 2.1 and Fig. 2.6). Three out of 43 sampling dates of 'Flavorich' showed significant difference of LT_{50} between floral buds on 5 cm stems and 20 cm stems, with these sampling all occurred in March (Table 2.1). In general, excised floral buds of both cultivars appeared less cold hardy than floral buds attached to stems, regardless of stem sizes. Floral buds attached on 5 cm stems and 20 cm stems exhibited close LT_{50} values, and no consistent pattern in difference of cold hardiness between the two types were reported (Fig. 2.1 or Table 2.1).

For vegetative buds, excised vegetative buds were more susceptible to low temperatures than vegetative buds attached to either 5 cm stems or 20 cm stems, especially before acclimation was completed (Fig. 2.2 or Table 2.2). For stem tissues, although there were few dates that were significant differences reported for estimated cold hardiness between 5 cm and 20 cm stems, the differences were not consistent, and did not present a clear pattern (Fig. 2.3 or Table 2.3).

Effect of tissue types on artificial freezing test

Overall, floral buds were observed to be the least cold resistant among all three tissue types for both varieties using the artificial freezing test in the winter season of 2014-2015 and 2015-2016, especially after deacclimation, as shown in Fig. 2.4A-B,

2.4D-E or Table 2.4 (tissues on 5 cm stems) and Fig. 2.5A-B, 2.5D-E or Table 2.5 (tissues on 20 cm stems). During acclimation stage, estimated cold hardiness of floral buds and vegetative buds were close.

As for the winter season of 2016-2017, floral buds and vegetative buds of 'Elberta' showed similar LT_{50}, and stems, however, were rated as most vulnerable to cold damage from February 6 to March 13, 2017 (Figs. 2.4C and 2.5C or Table 2.4 and Table 2.5). Floral and vegetative buds of 'Flavorich' had similar LT_{50} as well, until later in the spring (March 20 and March 27, 2017). In addition, stem tissues of 'Flavorich' appeared to be cold hardier than the other two tissues, similar to previous years (Fig. 2.4F and 2.5F or Table 2.4 and Table 2.5). Similar trend was also noted on tissues attached to 20 cm stems (Fig. 2.5 or Table 2.5).

For excised floral and vegetative buds, floral buds were reported to be less cold hardy than vegetative buds after deacclimation started during late winter season of 2014-2015 and 2015-2016, (Fig. 2.6A-B, 2.6D-E or Table 2.6). Before deacclimation, LT_{50} of floral and vegetative buds were close. In the third season, LT_{50} of excised floral buds and vegetative buds were similar for both cultivars, with only few instances after deacclimation when floral buds were reported to be less cold hardy than vegetative buds (Fig. 2.6C and 2.6F or Table 2.6).

Acclimation and deacclimation in peach

Acclimation and deacclimation patterns were observed in attached floral buds LT_{50} of 'Elberta' and 'Flavorich' from October to March of all three seasons and were

clearly demonstrated using the artificial freezing test as demonstrated by LT_{50} averages of floral buds from 5 cm and 20 cm stems (Fig. 2.7 or Table 2.7).

During the 2014-2015 season, cold hardiness of 'Elberta' floral buds attached to stems developed from October reaching a peak by January 26, 2015, then buds started to lose cold hardiness around February 9, 2015 with some degree of fluctuation. In the second season, 'Elberta' floral buds attached to stems reached their highest cold hardiness on January 26, 2016 and with LT_{50} starting to increase around February 23, 2016, which is later than the first season. During the 2016-2017 season, the acclimation process of floral bud of 'Elberta' was finished quite early. Cold hardiness of floral buds attached to stems was the highest on December 12, 2016, and LT_{50} of floral buds was stable from November 21, 2016 to February 6, 2017. Deacclimation started slowly and gradually afterward but didn't completed (Table 2.7).

For 'Flavorich', artificial freezing test started later than 'Elberta' in the season of 2014-2015. Floral buds attached to stems of 'Flavorich' were observed to be the hardiest at the first sampling date of the first season, which was January 20, 2015. Cold hardiness of floral buds attached to stems decreased after that. In the second season, floral buds attached to stems attained their highest cold hardiness on February 2, 2016. Acclimation of floral buds completed around January 12, 2016, and deacclimation started around February 23, 2016, which is later than the first season. For the third season, floral buds attached to stems of 'Flavorich', similar to 'Elberta', were already acclimated around November 7, 2016, and started to deacclimate around January 23, 2017. The deacclimation process of 'Flavorich' floral buds went faster and more advanced (Fig. 2.7C or Table 2.7).

Comparison between 'Elberta' and 'Flavorich' varieties

In general, floral buds of 'Elberta' were hardier than those of 'Flavorich' as identified by the artificial freezing test. For the first season, sample collection of 'Flavorich' material started on January as mentioned previously in the Material and Methods section, therefore data of late fall and midwinter is only available from the second and third season.

In the first season, floral buds attached to stems of 'Flavorich' started to deacclimate after the first collection date. Floral buds attached to stems of 'Elberta', on the other hand, maintained their cold hardiness until February 9, 2015 (Fig. 2.7A or Table 2.7). In the first date of the second season, October 27, 2015, 'Flavorich' floral buds attached to stems tended to be more cold resistant than 'Elberta', although not significant, which might imply that 'Flavorich' acclimated earlier than 'Elberta'. Yet further study is needed to confirm this. From November to mid-January of the second season, differences between floral bud cold hardiness were small between the two varieties, within 0.2˚C. Floral buds attached to stems of 'Elberta' kept acclimating until January 26, 2016, when they achieved their peak in cold hardiness with an LT_{50} of -18.2˚C. After that, 'Elberta' remained hardy until February 15, 2016. Thereafter, LT_{50} started to increase toward 0 ˚C throughout time, signifying deacclimation of 'Elberta' floral buds. 'Flavorich' floral buds attached to stems reached their highest cold hardiness on February 2, 2016, when LT_{50} was -15.7˚C, which was at a warmer temperature than 'Elberta' LT_{50} for that date. After February 2 buds started to deacclimate. 'Flavorich' floral bud's cold hardiness tended to be lower than that of 'Elberta' floral buds (Fig. 2.7B and Table 2.7).

In the winter season of 2016-2017, LT_{50} of 'Elberta' and 'Flavorich' floral buds were somewhat similar from October 10, 2016 to February 13, 2017. Estimated cold hardiness of floral buds of both cultivars increased until November 21, 2016, and were relatively stable until February 13, 2017, when floral buds of 'Flavorich' exhibited distinct deacclimation. Cold hardiness of 'Elberta' floral buds attached to stems was maintained longer and the deacclimation process progressed slowly without reaching a clear inflection point (Fig. 2.7C).

Discussion

Peach is one of the most important fruit in Georgia. In 2015, Georgia produced 40,600 tons of peaches (USDA, 2016). In Georgia, traditionally the last freeze can occur late February or early March, when peach floral buds are the most susceptible to freezing temperatures. It is, therefore, important for peach growers to be informed of the cold hardiness of their crop in a timely and accurate fashion in advance of upcoming freezing events. Growers can use this information to estimate possible damage to plants and probable losses of yield and profit. Cold hardiness information can help growers to make freeze protection decisions. Thus, the knowledge of freezing injury and cold hardiness measurement in peaches are critical.

The standard test to measure cold hardiness of plants is the artificial freezing test. This method evaluates the direct damage caused by low temperatures, allowing buds to interact with other organs during freezing and recovery, therefore closely resembling field conditions. Critical bud temperature determined by artificial freezing test is considered an accurate estimation of cold hardiness of the crop in field conditions. This

method has been widely used on fruit crops (Proebsting Jr and Mills, 1978). However, yet as a standard test, artificial freezing tests can be affected by many different factors, such as sampling type, sample size, and collection time, all of which are rarely studied. Our current project characterized the effect of sampling types (tissues on 5 cm stems, tissues on 20 cm stems, and excised buds) in peach artificial freezing tests and how they affected cold hardiness evaluations.

In previous studies, sample excision produced a direct effect on water behavior and on the nucleating point of the freezing process. Floral bud excision was revealed to elevate freezing temperature of cell sap, which caused lethal damage in floral buds of blueberry (Flinn and Ashworth, 1994) and grape (*Vitis vinifera* L.) (Quamme, 1986). Studies on peach floral buds exhibited the role of stems as an ice sink (Ashworth, 1982, Gross et al., 1984). Stems draw water out of the developing flower, relocating ice formation and avoiding freeze damage inside floral buds during the freezing process. An intact structure, in specific stem attachment and viability, is critical for expression of cold resistance of crops and crucial for cold hardiness estimation.

Stem length was shown to influence water behavior in plants as well. A logarithmic relationship between sample fresh weight of stems and nucleation temperature were noted in both tomato and peach plants (Anderson and Ashworth, 1985, Ashworth and Davis, 1984, Ashworth et al., 1985). Decreased sample size reduces water content, lowers the probability of ice formation and decreases the temperature at which water freezes, therefore the temperature that freezing damage occurs (Ashworth, 1991). In the study of peach stems, Ashworth and Davis (1984) examined stems of different weights, from less than 1 g to 20 g, as well as stems of intact trees. They found that small

stems did not freeze until reaching a lower temperature than either long stems or intact trees, while stems of 20 g froze at the same temperature as stems of intact trees. Increasing stem length did not further change freezing temperature (Ashworth and Davis, 1984). Hence, stems no less than 20 g performed similar to stems of intact trees during the freezing process. However, the authors did not investigate how stem lengths affect water behavior of buds.

In our study, we demonstrated the effect of excision and stem length on artificial freezing test of peach (Figs. 2.1-3 or Table 2.1). Artificial freezing test conducted with excised floral bud tended to yield more conservative estimates of cold hardiness of peach floral buds (Fig. 2.1 or Table 2.1), in agreement with previous studies (Ashworth, 1982). Stem length did not affect determination of bud cold hardiness of attached buds nor did affect stem cold hardiness determination (Fig. 2.1-3 or Table 2.1). Quamme et al. (1995) proposed the presence of a barrier at the flower base that protects the developing reproductive tissues from ice propagation coming from the flower bud axis. Therefore, it is not surprising that the water behavior of the attached floral buds to stems was not affected by the length of the stem. It is also possible that the minimum stem size in this study (5 cm) reached the critical size of 20 g that did affect freezing behavior of the sample as compared to nature as determined by Ashworth and Davis (1984). Stems of 5 cm were already big enough to fulfill the role of water sink, and therefore increasing stem size did not change the water behavior anymore as shown with 20 cm stem samples. In conclusion, buds attached to stems no shorter than 5 cm were found to be the optimal size for artificial tests and they are recommended for future studies.

In our artificial freezing tests, differences between the two varieties evaluated, 'Elberta' and 'Flavorich', were identified (Fig 2.7). These differences can be attributed to the inherited differences present in their chill requirement. The dormancy period of plants can be modeled as the time needed by a plant to fulfill their chilling requirement to resume growth. Chilling requirement is the amount of coldness (temperature within a given threshold, generally hours below 7.2 °C and above 0 °C) required by flower and leaf buds in order to complete morphological development (particularly for reproductive organs) and break rest (Layne and Bassi, 2008). Chilling requirement of 'Elberta' peach cultivar is reported at 850 chill hours and 'Flavorich' chill requirement is reported at 650 chill hours. 'Elberta' trees need to experience longer periods of low temperatures in order to satisfy their chill requirement than 'Flavorich' trees. Once their chill is satisfied and warm temperatures occur, flower bud break will happen (Faust et al., 1997).

Differences in deacclimation patterns of 'Elberta' and 'Flavorich' were observed (Fig. 2.7 and Table 2.7). For 'Flavorich' floral buds, deacclimation started earlier than floral buds of 'Elberta' for all three seasons. In the first season, deacclimation process of 'Flavorich' already started after the first collection date (January 20, 2015), while 'Elberta' remained acclimated until February 9, 2016 (Fig. 2.7A and Table 2.7). LT_{50}s of 'Elberta' floral buds were always lower than that of 'Flavorich' floral buds. In the second season, floral buds of both cultivars started deacclimation around February 23, 2016. However, floral buds of 'Flavorich' did not achieve a critical bud temperature as low as 'Elberta'. During mid-winter, the lowest LT_{50} of 'Elberta' was -18.2 °C, and the lowest LT_{50} of 'Flavorich' was -15.5 °C (Table 2.7). For the 2016-2017 season, the Southeastern U.S. experienced an exceptional warm winter. By the end of this study on March 27,

2017, floral buds of ‘Elberta’ did not developed normally. Most of the floral buds were still tight or slightly swelling, with few open flowers on some trees. Vegetative buds did not sprout either. In the meantime, floral buds of ‘Flavorich’ were able to come out of dormancy, but bud development was slow and not uniform. As shown in the deacclimation pattern, ‘Elberta’ floral buds did not show a clear deacclimation during the third winter, and ‘Flavorich’ exhibited a gradual deacclimation (Fig. 2.7C and Table 2.7). During this time, difference between LT_{50} of ‘Elberta’ floral buds and ‘Flavorich’ floral buds was the greatest, which might have been contributed both by genetic differences of the two cultivars and difference in the bud developmental stage. In summary, the difference in critical bud temperature and deacclimation pattern of two cultivars is consistent with the fact that ‘Elberta’ has a higher chilling requirement than ‘Flavorich’, which indicates that the ‘Elberta’ peach plants were adapted to longer and colder winters than ‘Flavorich’ peach plants.

Cold hardiness in plants is affected by both the genetic potential of the crop and the environmental effect on its performance. Proebsting and Mills (1972) reported floral buds LT_{50} of ‘Elberta’ grown in Washington State to be lower than temperatures reported in our test. Ballard et al. (1999) also reported full bloom stages occurring on average around April 11 in Washington State, which is later than Georgia. Our study measured the critical bud temperature of peach floral buds of two representative cultivars of Georgia peach production, which are more suitable for Georgia than the cold hardiness references available from Washington State.

Overall, ‘Elberta’ floral buds acclimated to be able to sustain lower critical bud freezing temperatures than ‘Flavorich’, also overwinter ‘Flavorich’ floral buds

deacclimated earlier and faster than ‘Elberta’ floral buds for all seasons. From our results, the artificial freezing test was able to capture different cold hardiness levels and deacclimation patterns between ‘Elberta’ and ‘Flavorich’.

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Table 2.1. Sampling types effect on critical bud freezing temperatures (LT_{50}) estimations for floral buds using the artificial freezing test. LT_{50} were calculated for floral buds of 'Elberta' and 'Flavorich' for the winter seasons of 2014-2015, 2015-2016, and 2016-2017.

Date	Cultivar	5 cm stem		20 cm stem		Excised		Cultivar	5 cm stem		20 cm stem		Excised	
2014-2015 Season														
10/13/14	Elberta	-11.0[z]	NS	-10.0		-11.8		Flavorich	-[x]		-		-	
11/10/14	Elberta	-12.2	a[y]	-15.2	b	-15.0	ab	Flavorich	-		-		-	
12/01/14	Elberta	-16.9	ab	-18.7	b	-13.5	a	Flavorich	-		-		-	
01/12/15	Elberta	-16.1	b	-16.9	b	-10.2	a	Flavorich	-		-		-	
01/20/15	Elberta	-17.6		-20.2		-15.7		Flavorich	-17.6		-18.8		-17.2	
01/26/15	Elberta	-19.1	b	-19.9	b	-13.5	a	Flavorich	-15.4	b	-17.2	b	-9.0	a
02/02/15	Elberta	-16.9		-15.4		-12.8		Flavorich	-14.3	b	-15.0	b	-6.3	a
02/09/15	Elberta	-17.0		-17.6		-18.0		Flavorich	-13.1		-13.5		-11.3	
02/16/15	Elberta	-11.3		-13.1		-13.5		Flavorich	-13.2	b	-12.8	b	-5.5	a
02/23/15	Elberta	-13.8		-14.2		-13.5		Flavorich	-7.6		-12.0		-9.1	
03/02/15	Elberta	-10.1	b	-4.8	ab	1.8	a	Flavorich	0.0		0.9		2.5	
03/09/15	Elberta	-13.5		-12.8		-11.3		Flavorich	-11.3	b	-7.5	a	-7.5	a
03/16/15	Elberta	-11.3	a	-10.5	a	-13.5	b	Flavorich	-		-		-	
2015-2016 Season														
10/27/15	Elberta	-11.2	c	-9.0	b	-6.5	a	Flavorich	-12.7		-10.5		-9.8	
11/16/15	Elberta	-14.5		-14.6		-8.6		Flavorich	-14.6	b	-14.2	b	-7.5	a
12/01/15	Elberta	-11.6	ab	-13.4	b	-8.2	a	Flavorich	-11.1	ab	-13.5	b	-8.2	a
01/04/16	Elberta	-11.7		-15.0		-13.5		Flavorich	-12.7		-14.3		-12.0	
01/12/16	Elberta	-15.5		-14.6		-15.0		Flavorich	-15.8	b	-14.3	ab	-12.0	a
01/19/16	Elberta	-15.8		-15.4		-13.5		Flavorich	-14.1		-14.6		-12.8	
01/26/16	Elberta	-19.5	b	-16.9	ab	-14.6	a	Flavorich	-15.4	b	-15.0	b	-10.5	a
02/02/16	Elberta	-16.1		-15.7		-13.5		Flavorich	-16.1	b	-15.4	ab	-13.5	a
02/09/16	Elberta	-16.1		-17.2		-13.5		Flavorich	-14.2	b	-15.0	b	-10.5	a
02/15/16	Elberta	-17.6	b	-16.1	b	-11.3	a	Flavorich	-15.4		-14.3		-12.0	
02/23/16	Elberta	-13.9		-14.6		-9.8		Flavorich	-10.9		-10.5		-10.5	
03/01/16	Elberta	-12.0		-10.1		-9.8		Flavorich	-9.6		-9.8		-9.7	
03/07/16	Elberta	-10.1		-10.5		-7.5		Flavorich	-8.6	a	-10.9	b	-9.8	ab

03/09/16	Elberta	-8.6		-10.1		-7.5		Flavorich	-9.4	ab	-8.3	a	-10.5	ab
03/15/16	Elberta	-9.8		-9.8		-9.0		Flavorich	-8.3		-7.5		-7.5	
03/22/16	Elberta	-9.4		-9.8		-9.8		Flavorich	-9.4	b	-7.1	a	-9.8	b
03/29/16	Elberta	-8.3		-7.1		-7.5		Flavorich	-8.3		-4.4		-9.7	
04/05/16	Elberta	-8.6		-6.4		-8.2		Flavorich	-4.4	a	-6.0	ab	-8.3	b
2016-2017 Season														
10/10/16	Elberta	-12.8	ab	-14.6	b	-10.5	a	Flavorich	-16.1		-15.4		-12.0	
10/24/16	Elberta	-13.5	b	-12.8	ab	-8.3	a	Flavorich	-13.7		-15.7		-11.3	
11/07/16	Elberta	-16.5	b	-16.1	b	-11.3	a	Flavorich	-16.7	b	-17.6	b	-9.4	a
11/21/16	Elberta	-17.2	b	-17.2	b	-12.0	a	Flavorich	-18.8	b	-19.1	b	-13.5	a
12/11/16	Elberta	-19.1		-19.1		-17.3		Flavorich	-18.0	b	-18.7	b	-13.5	a
01/09/17	Elberta	-19.5		-17.6		-18.7		Flavorich	-19.5		-17.6		-18.7	
01/15/17	Elberta	-18.4		-16.9		-18.7		Flavorich	-19.1	b	-18.4	b	-15.7	a
01/23/17	Elberta	-16.5		-17.6		-16.5		Flavorich	-16.1		-16.1		-13.5	
01/30/17	Elberta	-16.5	ab	-18.7	b	-14.2	a	Flavorich	-16.5		-16.5		-16.5	
02/06/17	Elberta	-18.4	b	-18.4	b	-15.0	a	Flavorich	-15.9		-16.9		-13.5	
02/13/17	Elberta	-16.5		-16.5		-15.0		Flavorich	-15.4		-16.1		-13.5	
02/20/17	Elberta	-16.5		-17.2		-15.7		Flavorich	-15.0	b	-14.2	ab	-12.0	a
02/27/17	Elberta	-15.7	ab	-16.9	b	-11.3	a	Flavorich	-13.1		-12.4		-10.5	
03/06/17	Elberta	-17.2		-15.4		-15.0		Flavorich	-12.0		-9.8		-10.5	
03/13/17	Elberta	-15.7		-15.4		-12.7		Flavorich	-10.5		-7.9		-8.3	
03/20/17	Elberta	-15.0		-13.1		-15.0		Flavorich	-5.2		-5.7		-5.9	
03/27/17	Elberta	-15.4		-14.6		-14.2		Flavorich	-7.5		-6.8		-9.0	

[z]Results shown in table represents the average value of eight replicates.

[y]Different letters within a row and within a variety represent statistically significant differences ($P<0.05$) for LT_{50} of floral buds between different sampling types as determined by Student's *t* test.

[x]Missing data represents dates in which artificial freezing tests were not performed.

[NS]No statistical significant differences expressed as missing mean separation within a row and within a variety.

Table 2.2. Sampling types effect on critical bud freezing temperatures (LT_{50}) estimations for vegetative buds using the artificial freezing test. LT_{50} were calculated for vegetative buds of 'Elberta' and 'Flavorich' for the winter seasons of 2014-2015, 2015-2016, and 2016-2017.

Date	Cultivar	5 cm stem		20 cm stem		Excised		Cultivar	5 cm stem		20 cm stem		Excised	
2014-2015 Season														
10/13/14	Elberta	-8.1[z]	NS	-5.1		-5.3		Flavorich	-[x]		-		-	
11/10/14	Elberta	-12.7		-17.4		-14.6		Flavorich	-		-		-	
12/01/14	Elberta	-16.6		-19.5		-13.5		Flavorich	-		-		-	
01/12/15	Elberta	-15.7		-13.4		-9.0		Flavorich	-		-		-	
01/20/15	Elberta	-17.2		-16.5		-16.5		Flavorich	-17.2		-18.0		-14.3	
01/26/15	Elberta	-18.0	b[y]	-18.8	b	-12.0	a	Flavorich	-15.0		-13.5		-12.0	
02/02/15	Elberta	-11.3		-12.8		-10.5		Flavorich	-9.8		-9.0		-8.5	
02/09/15	Elberta	-18.0		-18.8		-16.5		Flavorich	-12.8		-15.8		-11.3	
02/16/15	Elberta	-15.8		-15.0		-12.0		Flavorich	-16.5		-15.0		-12.0	
02/23/15	Elberta	-21.0		-22.9		-21.4		Flavorich	-20.2		-18.7		-19.5	
03/02/15	Elberta	-19.9		-19.6		-15.1		Flavorich	-12.6		-6.5		-2.5	
03/09/15	Elberta	-16.5		-14.3		-17.2		Flavorich	-9.8		-10.5		-9.0	
03/16/15	Elberta	-10.5		-10.5		-10.5		Flavorich	-		-		-	
2015-2016 Season														
10/27/15	Elberta	-10.5	b	-9.0	b	-4.4	a	Flavorich	-11.1		-11.2		-9.8	
11/16/15	Elberta	-14.8		-15.5		-10.5		Flavorich	-12.0	b	-13.5	b	-7.1	a
12/01/15	Elberta	-17.2	b	-16.5	b	-10.5	a	Flavorich	-14.6		-15.8		-8.9	
01/04/16	Elberta	-16.5		-15.7		-16.9		Flavorich	-11.3		-16.5		-13.5	
01/12/16	Elberta	-16.5		-19.5		-16.5		Flavorich	-17.3	b	-15.8	ab	-13.5	a
01/19/16	Elberta	-19.5		-20.2		-16.9		Flavorich	-18.0		-15.8		-17.3	
01/26/16	Elberta	-21.7		-20.2		-22.2		Flavorich	-19.5		-18.8		-18.5	
02/02/16	Elberta	-21.8		-21.7		-19.5		Flavorich	-19.5		-18.7		-18.5	
02/09/16	Elberta	-21.7		-21.8		-20.2		Flavorich	-21.7		-20.2		-18.7	
02/15/16	Elberta	-21.7	b	-21.7	b	-15.7	a	Flavorich	-21.0		-18.8		-18.0	
02/23/16	Elberta	-19.5		-20.2		-18.7		Flavorich	-20.2		-19.5		-17.3	
03/01/16	Elberta	-18.7		-15.8		-18.8		Flavorich	-17.3		-17.3		-14.2	
03/07/16	Elberta	-18.0	b	-15.0	ab	-11.3	a	Flavorich	-12.8		-12.8		-12.8	

03/09/16	Elberta	-16.5	b	-17.3	b	-13.5	a	Flavorich	-10.5	b	-11.3	b	-7.5	a
03/15/16	Elberta	-15.0	b	-10.5	a	-10.5	a	Flavorich	-12.0	a	-13.5	b	-13.5	b
03/22/16	Elberta	-13.5	ab	-15.0	b	-12.7	a	Flavorich	-15.0		-13.5		-14.2	
03/29/16	Elberta	-13.5		-13.5		-12.7		Flavorich	-9.7		-13.5		-13.5	
04/05/16	Elberta	-16.5	b	-15.7	b	-13.5	a	Flavorich	-16.0	b	-12.7	a	-12.7	a
2016-2017 Season														
10/10/16	Elberta	-13.5		-12.0		-10.5		Flavorich	-12.8	b	-14.3	b	-8.3	a
10/24/16	Elberta	-11.3	b	-11.3	b	-7.5	a	Flavorich	-12.7		-12.0		-10.5	
11/07/16	Elberta	-14.2	b	-14.2	b	-10.1	a	Flavorich	-12.0	ab	-15.0	b	-8.3	a
11/21/16	Elberta	-15.7		-16.5		-15.7		Flavorich	-18.4	b	-15.7	b	-10.5	a
12/11/16	Elberta	-18.1		-18.7		-18.0		Flavorich	-19.1	b	-19.5	b	-12.0	a
01/09/17	Elberta	-18.8		-19.5		-20.2		Flavorich	-18.8		-19.5		-20.2	
01/15/17	Elberta	-19.5		-21.7		-20.2		Flavorich	-17.2		-17.2		-17.2	
01/23/17	Elberta	-16.5		-20.2		-17.2		Flavorich	-15.7		-17.2		-15.7	
01/30/17	Elberta	-18.7		-18.0		-19.5		Flavorich	-15.0		-18.7		-16.5	
02/06/17	Elberta	-19.5		-20.2		-20.2		Flavorich	-15.7		-16.5		-12.8	
02/13/17	Elberta	-18.0		-20.2		-17.2		Flavorich	-15.7		-16.5		-15.0	
02/20/17	Elberta	-16.5		-18.7		-15.8		Flavorich	-15.7	b	-15.0	b	-11.3	a
02/27/17	Elberta	-18.0		-15.7		-15.0		Flavorich	-11.3		-13.5		-9.8	
03/06/17	Elberta	-16.5		-18.7		-15.0		Flavorich	-12.0		-12.7		-12.0	
03/13/17	Elberta	-15.0		-15.7		-16.5		Flavorich	-12.0		-9.8		-10.5	
03/20/17	Elberta	-18.7		-19.5		-18.7		Flavorich	-10.5	b	-11.3	b	-8.2	a
03/27/17	Elberta	-18.0		-19.5		-18.7		Flavorich	-11.3		-10.5		-11.2	

[z]Results shown in table represents the average value of four replicates.

[y]Different letters within a row and within a variety represent statistically significant differences ($P<0.05$) for LT_{50} of vegetative buds between different sampling types as determined by Student's *t* test.

[x]Missing data represents dates in which artificial freezing tests were not performed.

[NS]No statistical significant differences expressed as missing mean separation within a row and within a variety.

Table 2.3. Sampling types effect on critical freezing temperatures (LT_{50}) estimations for stem tissues using the artificial freezing test. LT_{50} were calculated for stem tissues of 'Elberta' and 'Flavorich' for winter of 2014-15, 2015-16, and 2016-17.

Date	Cultivar	5 cm stem		20 cm stem		Cultivar	5 cm stem		20 cm stem	
2014-2015 Season										
10/13/14	Elberta	-10.5[z]	NS	-10.5		Flavorich	-[x]		-	
11/10/14	Elberta	-13.5		-13.5		Flavorich	-		-	
12/01/14	Elberta	-20.2		-18.8		Flavorich	-		-	
01/12/15	Elberta	-19.5		-21.7		Flavorich	-		-	
01/20/15	Elberta	-21.0		-20.2		Flavorich	-21.0		-20.2	
01/26/15	Elberta	-22.5		-24.0		Flavorich	-21.0		-22.5	
02/02/15	Elberta	-22.5		-21.0		Flavorich	-22.5		-22.5	
02/09/15	Elberta	-21.8		-22.5		Flavorich	-20.2		-21.0	
02/16/15	Elberta	-22.5		-22.5		Flavorich	-22.5		-21.7	
02/23/15	Elberta	-22.5		-24.0		Flavorich	-20.2	a	-22.5	b
03/02/15	Elberta	-24.3	b[y]	-22.9	a	Flavorich	-20.2	b	-13.5	a
03/09/15	Elberta	-20.7		-19.5		Flavorich	-16.5		-16.5	
03/16/15	Elberta	-22.5		-21.0		Flavorich	-		-	
2015-2016 Season										
10/27/15	Elberta	-10.5		-10.5		Flavorich	-11.2	b	-8.3	a
11/16/15	Elberta	-12.0		-12.0		Flavorich	-14.1		-16.5	
12/01/15	Elberta	-15.7		-14.2		Flavorich	-15.8		-16.5	
01/04/16	Elberta	-15.0		-15.7		Flavorich	-17.3		-18.0	
01/12/16	Elberta	-21.7		-20.2		Flavorich	-22.5		-18.0	
01/19/16	Elberta	-22.1		-23.7		Flavorich	-18.0		-20.2	
01/26/16	Elberta	-23.2		-24.0		Flavorich	-20.2		-18.7	
02/02/16	Elberta	-18.8		-21.4		Flavorich	-22.5		-22.5	
02/09/16	Elberta	-24.0		-19.5		Flavorich	-20.2		-21.7	
02/15/16	Elberta	-22.2		-21.0		Flavorich	-18.7		-21.7	
02/23/16	Elberta	-18.0		-20.3		Flavorich	-21.0		-17.3	
03/01/16	Elberta	-18.0		-15.8		Flavorich	-21.0		-14.8	
03/07/16	Elberta	-18.0		-15.4		Flavorich	-15.8		-15.7	
03/09/16	Elberta	-12.8	a	-17.3	b	Flavorich	-15.8		-15.0	
03/15/16	Elberta	-15.8		-13.5		Flavorich	-13.5		-12.0	
03/22/16	Elberta	-15.0		-13.5		Flavorich	-13.5		-13.5	
03/29/16	Elberta	-13.5		-12.0		Flavorich	-12.7		-13.5	
04/05/16	Elberta	-16.5	b	-14.2	a	Flavorich	-13.6		-12.7	
2016-2017 Season										
10/10/16	Elberta	-11.3		-10.5		Flavorich	-11.3	a	-13.5	b
10/24/16	Elberta	-10.5		-10.5		Flavorich	-12.0		-12.0	
11/07/16	Elberta	-11.3		-12.0		Flavorich	-15.7		-15.7	
11/21/16	Elberta	-13.5		-13.5		Flavorich	-18.0		-16.5	
12/11/16	Elberta	-18.0		-16.5		Flavorich	-19.5		-18.7	
01/09/17	Elberta	-21.7		-19.6		Flavorich	-21.7		-19.6	

01/15/17	Elberta	-19.5	-18.0	Flavorich	-18.7	-21.7
01/23/17	Elberta	-18.7	-21.0	Flavorich	-18.7	-18.7
01/30/17	Elberta	-23.2	-21.7	Flavorich	-22.5	-21.0
02/06/17	Elberta	-18.0	-18.7	Flavorich	-18.0	-18.7
02/13/17	Elberta	-14.2	-12.8	Flavorich	-18.0	-18.0
02/20/17	Elberta	-12.7	-15.7	Flavorich	-18.0	-16.5
02/27/17	Elberta	-12.0	-13.5	Flavorich	-16.5	-16.5
03/06/17	Elberta	-10.5	-13.5	Flavorich	-13.5	-14.2
03/13/17	Elberta	-13.5	-14.9	Flavorich	-15.7	-16.5
03/20/17	Elberta	-16.5	-17.2	Flavorich	-14.2	-12.7
03/27/17	Elberta	-18.0	-17.2	Flavorich	-14.2	-13.5

[z]Results shown in table represents the average value of four replicates.
[y]Different letters within a row and within a variety represent statistically significant differences ($P<0.05$) for LT_{50} of stems between different sampling types as determined by Student's *t* test.
[x]Missing data represents dates in which artificial freezing tests were not performed.
[NS]No statistical significant differences expressed as missing mean separation within a row and within a variety.

Table 2.4. Tissue types effect on critical freezing temperature (LT_{50}) estimations for tissues on 5 cm stems. LT_{50} were calculated for floral buds, vegetative buds, and stems of 'Elberta' and 'Flavorich' for winter of 2014-2015, 2015-2016, and 2016-2017.

Date	Cultivar	Floral buds		Vegetative buds		Stems		Cultivar	Floral buds		Vegetative buds		Stems	
2014-2015 Season														
10/13/14	Elberta	-11.0[z]	NS	-8.1		-10.5		Flavorich	-[x]		-		-	
11/10/14	Elberta	-12.2		-12.7		-13.5		Flavorich	-		-		-	
12/01/14	Elberta	-16.9		-16.6		-20.2		Flavorich	-		-		-	
01/12/15	Elberta	-16.1		-15.7		-19.5		Flavorich	-		-		-	
01/20/15	Elberta	-17.6		-17.2		-21.0		Flavorich	-17.6		-17.2		-21.0	
01/26/15	Elberta	-19.1	ab[y]	-18.0	a	-22.5	b	Flavorich	-15.4	a	-15.0	a	-21.0	b
02/02/15	Elberta	-16.9	b	-11.3	a	-22.5	c	Flavorich	-14.3	b	-9.8	a	-22.5	c
02/09/15	Elberta	-17.0	a	-18.0	ab	-21.8	b	Flavorich	-13.1	a	-12.8	a	-20.2	b
02/16/15	Elberta	-11.3		-15.8		-22.5	b	Flavorich	-13.2	a	-16.5	ab	-22.5	b
02/23/15	Elberta	-13.8	a	-21.0	b	-22.5	b	Flavorich	-7.6	a	-20.2	b	-20.2	b
03/02/15	Elberta	-10.1	a	-19.9	b	-24.3	b	Flavorich	0.0	a	-12.6	b	-20.2	b
03/09/15	Elberta	-13.5	a	-16.5	ab	-20.7	b	Flavorich	-11.3	a	-9.8	a	-16.5	b
03/16/15	Elberta	-11.3	a	-10.5	a	-22.5	b	Flavorich	-		-		-	
2015-2016 Season														
10/27/15	Elberta	-11.2		-10.5		-10.5		Flavorich	-12.7		-11.1		-11.2	
11/16/15	Elberta	-14.5		-14.8		-12.0		Flavorich	-14.6		-12.0		-14.1	
12/01/15	Elberta	-11.6	a	-17.2	b	-15.7	b	Flavorich	-11.1		-14.6		-15.8	
01/04/16	Elberta	-11.7		-16.5		-15.0		Flavorich	-12.7	a	-11.3	a	-17.3	b
01/12/16	Elberta	-15.5	a	-16.5	a	-21.7	b	Flavorich	-15.8	a	-17.3	a	-22.5	b
01/19/16	Elberta	-15.8	a	-19.5	ab	-22.1	b	Flavorich	-14.1		-18.0		-18.0	
01/26/16	Elberta	-19.5	a	-21.7	ab	-23.2	b	Flavorich	-15.4	a	-19.5	ab	-20.2	b
02/02/16	Elberta	-16.1	a	-21.8	b	-18.8	ab	Flavorich	-16.1	a	-19.5	b	-22.5	c
02/09/16	Elberta	-16.1	a	-21.7	b	-24.0	b	Flavorich	-14.2	a	-21.7	b	-20.2	b
02/15/16	Elberta	-17.6	a	-21.7	ab	-22.2	b	Flavorich	-15.4	a	-21.0	b	-18.7	b
02/23/16	Elberta	-13.9	a	-19.5	b	-18.0	ab	Flavorich	-10.9	a	-20.2	b	-21.0	b
03/01/16	Elberta	-12.0	a	-18.7	b	-18.0	b	Flavorich	-9.6	a	-17.3	b	-21.0	b

03/07/16	Elberta	-10.1	a	-18.0	b	-18.0	b	Flavorich	-8.6	a	-12.8	b	-15.8	c
03/09/16	Elberta	-8.6	a	-16.5	c	-12.8	b	Flavorich	-9.4	a	-10.5	a	-15.8	b
03/15/16	Elberta	-9.8	a	-15.0	b	-15.8	b	Flavorich	-8.3	a	-12.0	b	-13.5	b
03/22/16	Elberta	-9.4	a	-13.5	b	-15.0	b	Flavorich	-9.4	a	-15.0	b	-13.5	b
03/29/16	Elberta	-8.3	a	-13.5	b	-13.5	b	Flavorich	-8.3		-9.7		-12.7	
04/05/16	Elberta	-8.6	a	-16.5	b	-16.5	b	Flavorich	-4.4	a	-16.0	b	-13.6	b
2016-2017 Season														
10/10/16	Elberta	-12.8		-13.5		-11.3		Flavorich	-16.1	b	-12.8	ab	-11.3	a
10/24/16	Elberta	-13.5		-11.3		-10.5		Flavorich	-13.7		-12.7		-12.0	
11/07/16	Elberta	-16.5	b	-14.2	ab	-11.3	a	Flavorich	-16.7	b	-12.0	a	-15.7	ab
11/21/16	Elberta	-17.2		-15.7		-13.5		Flavorich	-18.8		-18.4		-18.0	
12/11/16	Elberta	-19.1		-18.1		-18.0		Flavorich	-18.0		-19.1		-19.5	
01/09/17	Elberta	-19.5		-18.8		-21.7		Flavorich	-19.5		-18.8		-21.7	
01/16/17	Elberta	-18.4		-19.5		-19.5		Flavorich	-19.1		-17.2		-18.7	
01/23/17	Elberta	-16.5		-16.5		-18.7		Flavorich	-16.1	a	-15.7	a	-18.7	b
01/30/17	Elberta	-16.5	a	-18.7	a	-23.2	b	Flavorich	-16.5	a	-15.0	a	-22.5	b
02/06/17	Elberta	-18.4		-19.5		-18.0		Flavorich	-15.9		-15.7		-18.0	
02/13/17	Elberta	-16.5		-18.0		-14.2		Flavorich	-15.4		-15.7		-18.0	
02/20/17	Elberta	-16.5		-16.5		-12.7		Flavorich	-15.0	a	-15.7	ab	-18.0	b
02/27/17	Elberta	-15.7		-18.0		-12.0		Flavorich	-13.1		-11.3		-16.5	
03/06/17	Elberta	-17.2	b	-16.5	b	-10.5	a	Flavorich	-12.0		-12.0		-13.5	
03/13/17	Elberta	-15.7		-15.0		-13.5		Flavorich	-10.5	a	-12.0	ab	-15.7	b
03/20/17	Elberta	-15.0		-18.7		-16.5		Flavorich	-5.2	a	-10.5	b	-14.2	b
03/27/17	Elberta	-15.4		-18.0		-18.0		Flavorich	-7.5	a	-11.3	b	-14.2	c

[z]Results shown in table represents the average value of eight replicates for floral buds, and four replicates for vegetative buds and stem tissues.

[y] Different letters within a row and within a variety represent statistically significant differences ($P<0.05$) for LT_{50} of the different tissue types as determined by Student's *t* test.

[x]Missing data represents dates in which artificial freezing tests were not performed.

[NS]No statistical significant differences expressed as missing mean separation within a row and within a variety.

Table 2.5. Tissue types effect on critical freezing temperature (LT_{50}) estimations for tissues on 20 cm stems. LT_{50} were calculated for floral buds, vegetative buds, and stems of 'Elberta' and 'Flavorich' for winter of 2014-2015, 2015-2016, and 2016-2017.

Date	Cultivar	Floral buds		Vegetative buds		Stems		Cultivar	Floral buds		Vegetative buds		Stems	
2014-2015 Season														
10/13/14	Elberta	-10.0[z]	b	-5.1	a	-10.5	b	Flavorich	-[x]		-		-	
11/10/14	Elberta	-15.2	ab	-17.4	b	-13.5	a	Flavorich	-		-		-	
12/01/14	Elberta	-18.7	[NS]	-19.5		-18.8		Flavorich	-		-		-	
01/12/15	Elberta	-16.9	ab	-13.4	a	-21.7	b	Flavorich	-		-		-	
01/20/15	Elberta	-20.2	b	-16.5	a	-20.2	b	Flavorich	-18.8		-18.0		-20.2	
01/26/15	Elberta	-19.9	ab	-18.8	a	-24.0	b	Flavorich	-17.2	ab	-13.5	a	-22.5	b
02/02/15	Elberta	-15.4	a	-12.8	a	-21.0	b	Flavorich	-15.0	b	-9.0	a	-22.5	c
02/09/15	Elberta	-17.6	a	-18.8	ab	-22.5	b	Flavorich	-13.5	a	-15.8	ab	-21.0	b
02/16/15	Elberta	-13.1	a	-15.0	a	-22.5	b	Flavorich	-12.8	a	-15.0	a	-21.7	b
02/23/15	Elberta	-14.2	a	-22.9	b	-24.0	b	Flavorich	-12.0	a	-18.7	b	-22.5	b
03/02/15	Elberta	-4.8	a	-19.6	b	-22.9	b	Flavorich	0.9	a	-6.5	ab	-13.5	b
03/09/15	Elberta	-12.8	a	-14.3	a	-19.5	b	Flavorich	-7.5	a	-10.5	b	-16.5	c
03/16/15	Elberta	-10.5	a	-10.5	a	-21.0	b	Flavorich	-		-		-	
2015-2016 Season														
10/27/15	Elberta	-9.0		-9.0		-10.5		Flavorich	-10.5	ab	-11.2	b	-8.3	a
11/16/15	Elberta	-14.6		-15.5		-12.0		Flavorich	-14.2	ab	-13.5	a	-16.5	b
12/01/15	Elberta	-13.4		-16.5		-14.2		Flavorich	-13.5	a	-15.8	ab	-16.5	b
01/04/16	Elberta	-15.0		-15.7		-15.7		Flavorich	-14.3	a	-16.5	ab	-18.0	b
01/12/16	Elberta	-14.6	a	-19.5	b	-20.2	b	Flavorich	-14.3	a	-15.8	ab	-18.0	b
01/19/16	Elberta	-15.4	a	-20.2	b	-23.7	b	Flavorich	-14.6	a	-15.8	a	-20.2	b
01/26/16	Elberta	-16.9	a	-20.2	ab	-24.0	b	Flavorich	-15.0	a	-18.8	b	-18.7	b
02/02/16	Elberta	-15.7	a	-21.7	b	-21.4	b	Flavorich	-15.4	a	-18.7	b	-22.5	c
02/09/16	Elberta	-17.2		-21.8		-19.5		Flavorich	-15.0	a	-20.2	b	-21.7	b
02/15/16	Elberta	-16.1	a	-21.7	b	-21.0	b	Flavorich	-14.3	a	-18.8	b	-21.7	b
02/23/16	Elberta	-14.6	a	-20.2	b	-20.3	b	Flavorich	-10.5	a	-19.5	b	-17.3	b
03/01/16	Elberta	-10.1	a	-15.8	b	-15.8	b	Flavorich	-9.8	a	-17.3	b	-14.8	ab

03/07/16	Elberta	-10.5	a	-15.0	b	-15.4	b	Flavorich	-10.9	a	-12.8	ab	-15.7	b
03/09/16	Elberta	-10.1	a	-17.3	b	-17.3	b	Flavorich	-8.3	a	-11.3	ab	-15.0	b
03/15/16	Elberta	-9.8	a	-10.5	a	-13.5	b	Flavorich	-7.5	a	-13.5	b	-12.0	b
03/22/16	Elberta	-9.8	a	-15.0	b	-13.5	b	Flavorich	-7.1	a	-13.5	b	-13.5	b
03/29/16	Elberta	-7.1	a	-13.5	b	-12.0	b	Flavorich	-4.4	a	-13.5	b	-13.5	b
04/05/16	Elberta	-6.4	a	-15.7	b	-14.2	b	Flavorich	-6.0	a	-12.7	b	-12.7	b
2016-2017 Season														
10/10/16	Elberta	-14.6	b	-12.0	ab	-10.5	a	Flavorich	-15.4		-14.3		-13.5	
10/24/16	Elberta	-12.8		-11.3		-10.5		Flavorich	-15.7		-12.0		-12.0	
11/07/16	Elberta	-16.1		-14.2		-12.0		Flavorich	-17.6		-15.0		-15.7	
11/21/16	Elberta	-17.2	b	-16.5	ab	-13.5	a	Flavorich	-19.1	b	-15.7	a	-16.5	ab
12/11/16	Elberta	-19.1	a	-18.7	b	-16.5	b	Flavorich	-18.7		-19.5		-18.7	
01/09/17	Elberta	-17.6		-19.5		-19.6		Flavorich	-17.6		-19.5		-19.6	
01/16/17	Elberta	-16.9	a	-21.7	b	-18.0	ab	Flavorich	-18.4	a	-17.2	a	-21.7	b
01/23/17	Elberta	-17.6	a	-20.2	ab	-21.0	b	Flavorich	-16.1		-17.2		-18.7	
01/30/17	Elberta	-18.7		-18.0		-21.7		Flavorich	-16.5	a	-18.7	ab	-21.0	b
02/06/17	Elberta	-18.4		-20.2		-18.7		Flavorich	-16.9		-16.5		-18.7	
02/13/17	Elberta	-16.5	b	-20.2	c	-12.8	a	Flavorich	-16.1		-16.5		-18.0	
02/20/17	Elberta	-17.2		-18.7		-15.7		Flavorich	-14.2	a	-15.0	ab	-16.5	b
02/27/17	Elberta	-16.9		-15.7		-13.5		Flavorich	-12.4		-13.5		-16.5	
03/06/17	Elberta	-15.4		-18.7		-13.5		Flavorich	-9.8	a	-12.7	ab	-14.2	b
03/13/17	Elberta	-15.4		-15.7		-14.9		Flavorich	-7.9	a	-9.8	a	-16.5	b
03/20/17	Elberta	-13.1	a	-19.5	b	-17.2	ab	Flavorich	-5.7	a	-11.3	b	-12.7	b
03/27/17	Elberta	-14.6		-19.5		-17.2		Flavorich	-6.8	a	-10.5	b	-13.5	c

[z]Results shown in table represents the average value of eight replicates for floral buds, and four replicates for vegetative buds and stem tissues.

[y]Different letters within a row and within a variety represent statistically significant differences ($P<0.05$) for LT_{50} of the different tissue types as determined by Student's *t* test.

[x]Missing data represents dates in which artificial freezing tests were not performed.

[NS]No statistical significant differences expressed as missing mean separation within a row and within a variety.

Table 2.6. Tissue types effect on critical bud freezing temperatures (LT_{50}) estimations for excised buds using the artificial freezing test. LT_{50} were calculated for floral and vegetative buds of 'Elberta' and 'Flavorich' for the winter seasons of 2014-2015, 2015-2016, and 2016-2017.

Date	Cultivar	Floral buds		Vegetative buds		Cultivar	Floral buds		Vegetative b	
2014-2015 Season										
10/13/14	Elberta	-11.8[z]	b[y]	-5.3	a	Flavorich	-[x]		-	
11/10/14	Elberta	-15.0	NS	-14.6		Flavorich	-		-	
12/01/14	Elberta	-13.5		-13.5		Flavorich	-		-	
01/12/15	Elberta	-10.2		-9.0		Flavorich	-		-	
01/20/15	Elberta	-15.7		-16.5		Flavorich	-17.2		-14.3	
01/26/15	Elberta	-13.5		-12.0		Flavorich	-9.0		-12.0	
02/02/15	Elberta	-12.8		-10.5		Flavorich	-6.3		-8.5	
02/09/15	Elberta	-18.0		-16.5		Flavorich	-11.3		-11.3	
02/16/15	Elberta	-13.5		-12.0		Flavorich	-5.5		-12.0	
02/23/15	Elberta	-13.5	a	-21.4	b	Flavorich	-9.1	a	-19.5	b
03/02/15	Elberta	1.8	a	-15.1	b	Flavorich	2.5		-2.5	
03/09/15	Elberta	-11.3	a	-17.2	b	Flavorich	-7.5		-9.0	
03/16/15	Elberta	-13.5	b	-10.5	a	Flavorich	-		-	
2015-2016 Season										
10/27/15	Elberta	-6.5		-4.4		Flavorich	-9.8		-9.8	
11/16/15	Elberta	-8.6		-10.5		Flavorich	-7.5		-7.1	
12/01/15	Elberta	-8.2		-10.5		Flavorich	-8.2		-8.9	
01/04/16	Elberta	-13.5		-16.9		Flavorich	-12.0		-13.5	
01/12/16	Elberta	-15.0		-16.5		Flavorich	-12.0		-13.5	
01/19/16	Elberta	-13.5		-16.9		Flavorich	-12.8		-17.3	
01/26/16	Elberta	-14.6		-22.2		Flavorich	-10.5	a	-18.5	b
02/02/16	Elberta	-13.5		-19.5		Flavorich	-13.5	a	-18.5	b
02/09/16	Elberta	-13.5		-20.2		Flavorich	-10.5		-18.7	
02/15/16	Elberta	-11.3		-15.7		Flavorich	-12.0	a	-18.0	b
02/23/16	Elberta	-9.8	a	-18.7	b	Flavorich	-10.5	a	-17.3	b
03/01/16	Elberta	-9.8	a	-18.8	b	Flavorich	-9.7		-14.2	
03/07/16	Elberta	-7.5		-11.3		Flavorich	-9.8		-12.8	
03/09/16	Elberta	-7.5	a	-13.5	b	Flavorich	-10.5	b	-7.5	a
03/15/16	Elberta	-9.0		-10.5		Flavorich	-7.5	a	-13.5	b
03/22/16	Elberta	-9.8	a	-12.7	b	Flavorich	-9.8	a	-14.2	b
03/29/16	Elberta	-7.5	a	-12.7	b	Flavorich	-9.7	a	-13.5	b
04/05/16	Elberta	-8.2	a	-13.5	b	Flavorich	-8.3	a	-12.7	b
2016-2017 Season										
10/10/16	Elberta	-10.5		-10.5		Flavorich	-12.0		-8.3	
10/24/16	Elberta	-8.3		-7.5		Flavorich	-11.3		-10.5	
11/07/16	Elberta	-11.3		-10.1		Flavorich	-9.4		-8.3	
11/21/16	Elberta	-12.0		-15.7		Flavorich	-13.5		-10.5	
12/11/16	Elberta	-17.3		-18.0		Flavorich	-13.5		-12.0	

01/09/17	Elberta	-18.7		-20.2		Flavorich	-18.7		-20.2		
01/15/17	Elberta	-18.7		-20.2		Flavorich	-15.7		-17.2		
01/23/17	Elberta	-16.5		-17.2		Flavorich	-13.5		-15.7		
01/30/17	Elberta	-14.2		-19.5		Flavorich	-16.5		-16.5		
02/06/17	Elberta	-15.0	a	-20.2	b	Flavorich	-13.5		-12.8		
02/13/17	Elberta	-15.0		-17.2		Flavorich	-13.5		-15.0		
02/20/17	Elberta	-15.7		-15.8		Flavorich	-12.0		-11.3		
02/27/17	Elberta	-11.3		-15.0		Flavorich	-10.5		-9.8		
03/06/17	Elberta	-15.0		-15.0		Flavorich	-10.5		-12.0		
03/13/17	Elberta	-12.7	a	-16.5	b	Flavorich	-8.3	a	-10.5	b	
03/20/17	Elberta	-15.0		-18.7		Flavorich	-5.9		-8.2		
03/27/17	Elberta	-14.2		-18.7		Flavorich	-9.0		-11.2		

[z]Results shown in table represents the average value of four replicates.
[y]Different letters within a row and within a variety represent statistically significant differences ($P<0.05$) for LT_{50} of different tissue types as determined by Student's *t* test.
[x]Missing data represents dates in which artificial freezing tests were not performed.
[NS]No statistical significant differences expressed as missing mean separation within a row and within a variety.

Table 2.7. Critical bud temperatures (LT_{50}) were calculated for floral buds attached to stems of 'Elberta' and 'Flavorich' for the winter seasons of 2014-2015, 2015-2016, and 2016-2017.

Date	Cultivar	LT_{50}		Bud stage[x]	Cultivar	LT_{50}		Bud stage
2014-2015 Season								
10/13/14	Elberta	-10.5	b [zy]		Flavorich	-[w]		
11/10/14	Elberta	-13.7	cde		Flavorich	-		
12/01/14	Elberta	-17.8	ghi		Flavorich	-		
01/12/15	Elberta	-16.5	fgh		Flavorich	-		
01/20/15	Elberta	-18.9	hi		Flavorich	-18.2	e	
01/26/15	Elberta	-19.5	i		Flavorich	-16.3	de	
02/02/15	Elberta	-16.1	efg		Flavorich	-14.6	cd	
02/09/15	Elberta	-17.3	ghi		Flavorich	-13.3	c	
02/16/15	Elberta	-12.2	bcd		Flavorich	-13.0	c	
02/23/15	Elberta	-14.0	def		Flavorich	-9.5	b	
03/02/15	Elberta	-7.4	a		Flavorich	0.4	a	
03/09/15	Elberta	-13.1	bcd		Flavorich	-9.4	b	
03/16/15	Elberta	-11.0	bc		Flavorich	-		
2015-2016 Season								
10/27/15	Elberta	-9.7	ab	Tight bud	Flavorich	-11.6	fg	Tight bud
11/16/15	Elberta	-14.6	efgh	Tight bud	Flavorich	-14.4	ij	Tight bud
12/01/15	Elberta	-12.5	cde	Tight bud	Flavorich	-12.3	gh	Tight bud
01/04/16	Elberta	-13.4	def	Tight bud	Flavorich	-13.5	hi	Tight bud
01/12/16	Elberta	-15.1	fgh	Tight bud	Flavorich	-15.0	ij	Tight bud
01/19/16	Elberta	-15.6	fgh	Tight bud	Flavorich	-14.3	ij	Tight bud
01/26/16	Elberta	-18.2	i	Tight bud	Flavorich	-15.2	ij	Tight bud
02/02/16	Elberta	-15.9	ghi	Tight bud	Flavorich	-15.7	j	Tight bud
02/09/16	Elberta	-16.7	hi	Tight bud	Flavorich	-14.6	ij	Tight bud
02/15/16	Elberta	-16.9	hi	Tight bud	Flavorich	-14.8	ij	Tight bud
02/23/16	Elberta	-14.3	efg	Tight bud	Flavorich	-10.7	efg	Tight bud
03/01/16	Elberta	-11.1	bcd	Tight bud	Flavorich	-9.7	cde	Bud swell
03/07/16	Elberta	-10.3	bc	Bud swell	Flavorich	-9.8	def	Green bud
03/09/16	Elberta	-9.4	ab	Green bud	Flavorich	-8.8	cd	Pink
03/15/16	Elberta	-9.8	ab	Bloom	Flavorich	-7.9	bc	Shuck split
03/22/16	Elberta	-9.6	ab	Petal fall	Flavorich	-8.3	cd	Shuck split
03/29/16	Elberta	-7.7	a	Petal fall	Flavorich	-6.3	ab	Shuck off
04/05/16	Elberta	-7.5	a	Shuck split	Flavorich	-5.2	a	Shuck off
2016-2017 Season								
10/10/16	Elberta	-13.7	ab	Tight bud	Flavorich	-15.7	fg	Tight bud
10/24/16	Elberta	-13.1	a	Tight bud	Flavorich	-14.7	ef	Tight bud
11/07/16	Elberta	-16.3	cde	Tight bud	Flavorich	-17.1	ghij	Tight bud
11/21/16	Elberta	-17.2	cdefg	Tight bud	Flavorich	-18.9	j	Tight bud
12/11/16	Elberta	-19.1	g	Tight bud	Flavorich	-18.4	ij	Tight bud
01/09/17	Elberta	-18.6	fg	Tight bud	Flavorich	-18.2	hij	Tight bud

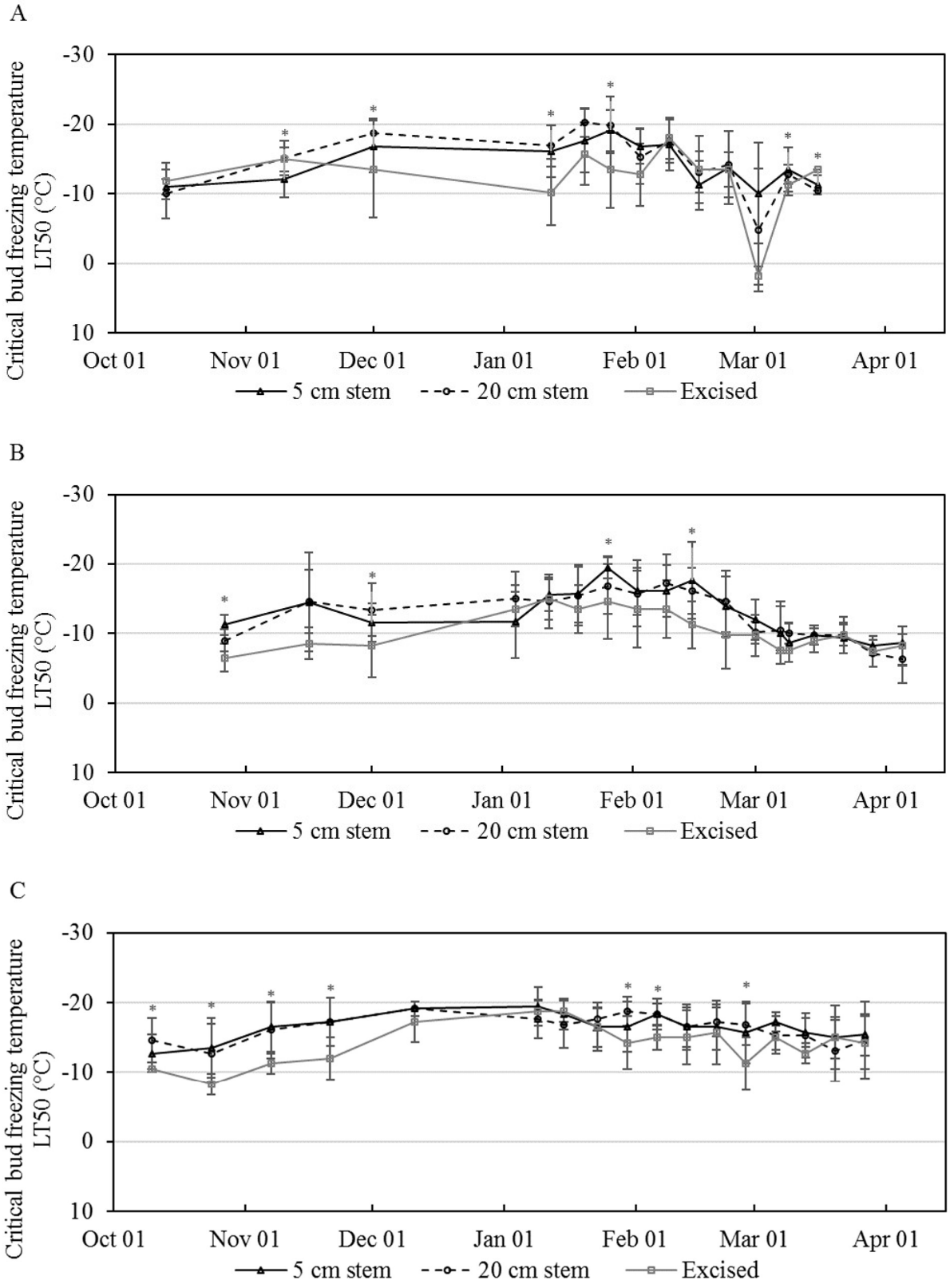
01/15/17	Elberta	-17.6	defg	Tight bud	Flavorich	-18.7	j	Tight bud
01/23/17	Elberta	-17.1	cdefg	Tight bud	Flavorich	-16.1	fgh	Tight bud
01/30/17	Elberta	-17.6	defg	Tight bud	Flavorich	-16.5	fghi	Tight bud
02/06/17	Elberta	-18.4	efg	Tight bud	Flavorich	-16.4	fghi	Tight bud
02/13/17	Elberta	-16.5	cdef	Tight bud	Flavorich	-15.7	fg	Tight bud
02/20/17	Elberta	-16.9	cdef	Tight bud	Flavorich	-14.6	ef	Bud swell
02/27/17	Elberta	-16.3	cde	Tight bud	Flavorich	-12.7	de	Bud swell
03/06/17	Elberta	-16.3	cde	Tight bud	Flavorich	-10.9	cd	Bud swell
03/13/17	Elberta	-15.6	bcd	Tight bud	Flavorich	-9.2	bc	Green bud
03/20/17	Elberta	-14.1	ab	Tight bud	Flavorich	-5.5	a	Green bud
03/27/17	Elberta	-15.0	abc	Tight bud	Flavorich	-7.1	ab	Bloom

[z]Results shown in table represents the average value of 16 reps across 5 cm stems and 20 cm stems.

[y]Different letters within a column and within a season represent statistically significant differences ($P<0.05$) for LT_{50} of attached floral buds as determined by Student's *t* test.

[x]Most prominent floral bud development stage per date across samples (Horton and Johnson, 2005). Data were not collected for winter of 2014-2015.

[w]Symbol – represents dates in which the artificial freezing tests were not performed


A
B
C
Critical bud freezing temperature LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
5 cm stem
20 cm stem
Excised

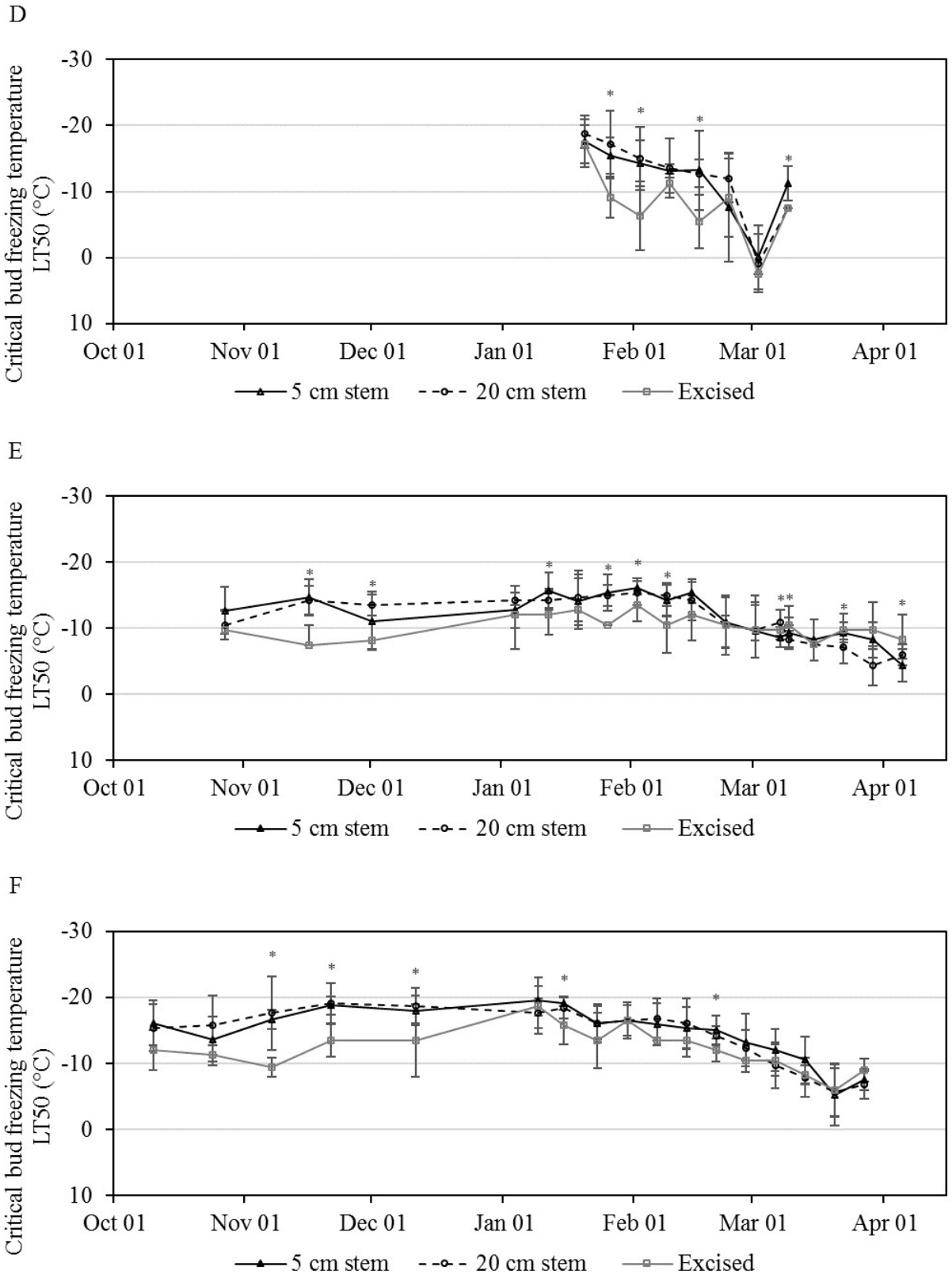
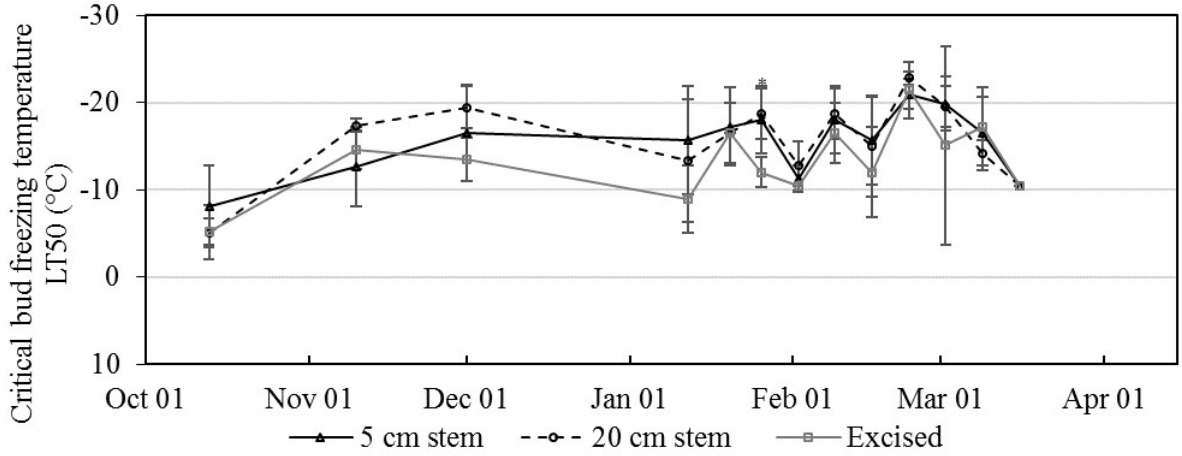


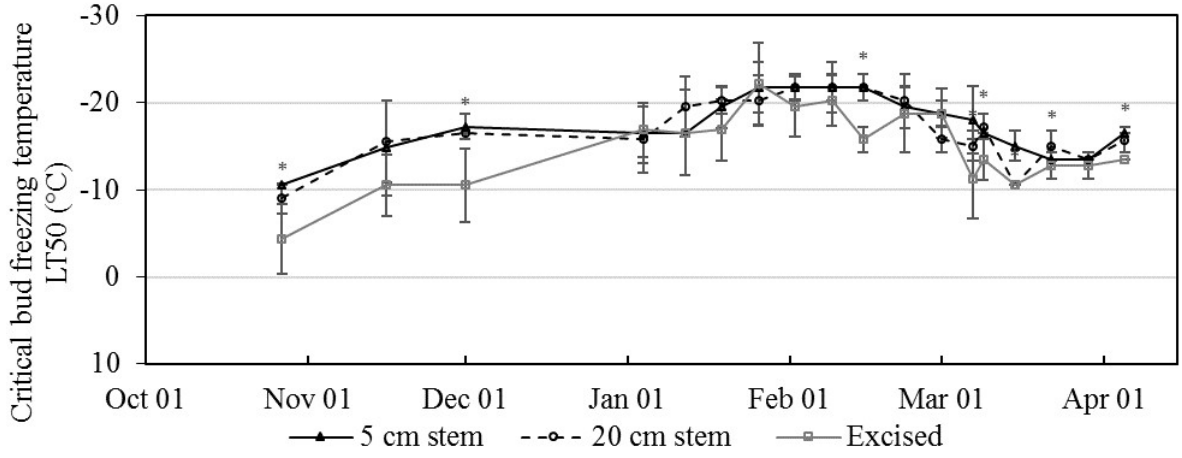
Fig. 2.1. Sampling types effect on critical bud freezing temperatures (LT_{50}) estimations for floral buds using the artificial freezing test. LT_{50} were calculated for floral buds of

‘Elberta’ (A-C) and ‘Flavorich’ (D-F) for winter of 2014-2015 (A, D), 2015-2016 (B, E), and 2016-2017 (C, F). Statistical significant differences (P<0.05) determined by Student’s t test for LT_{50} across different sampling types are indicated by asterisks. Bars indicate standard deviations.

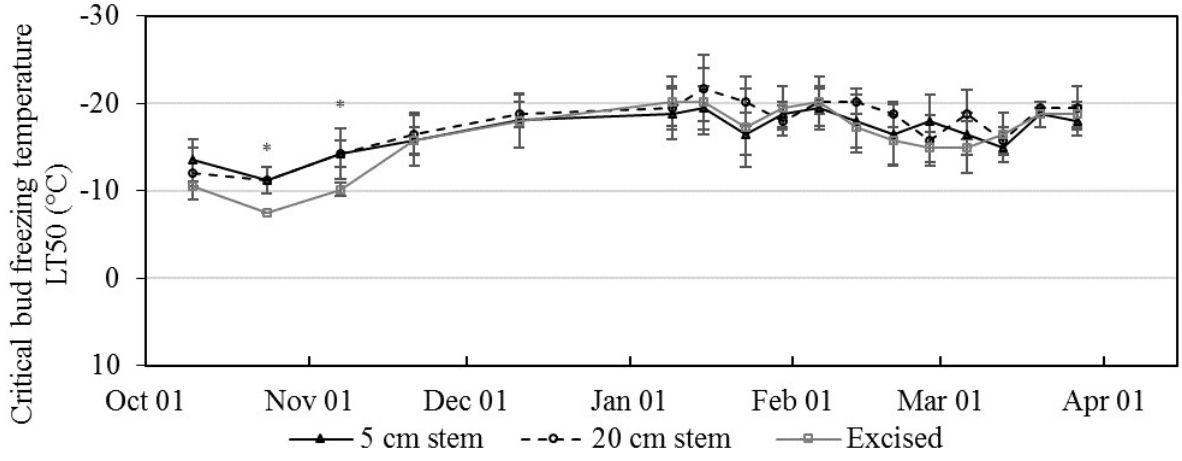
A


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
5 cm stem
20 cm stem
Excised

B


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
5 cm stem
20 cm stem
Excised

C


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
5 cm stem
20 cm stem
Excised

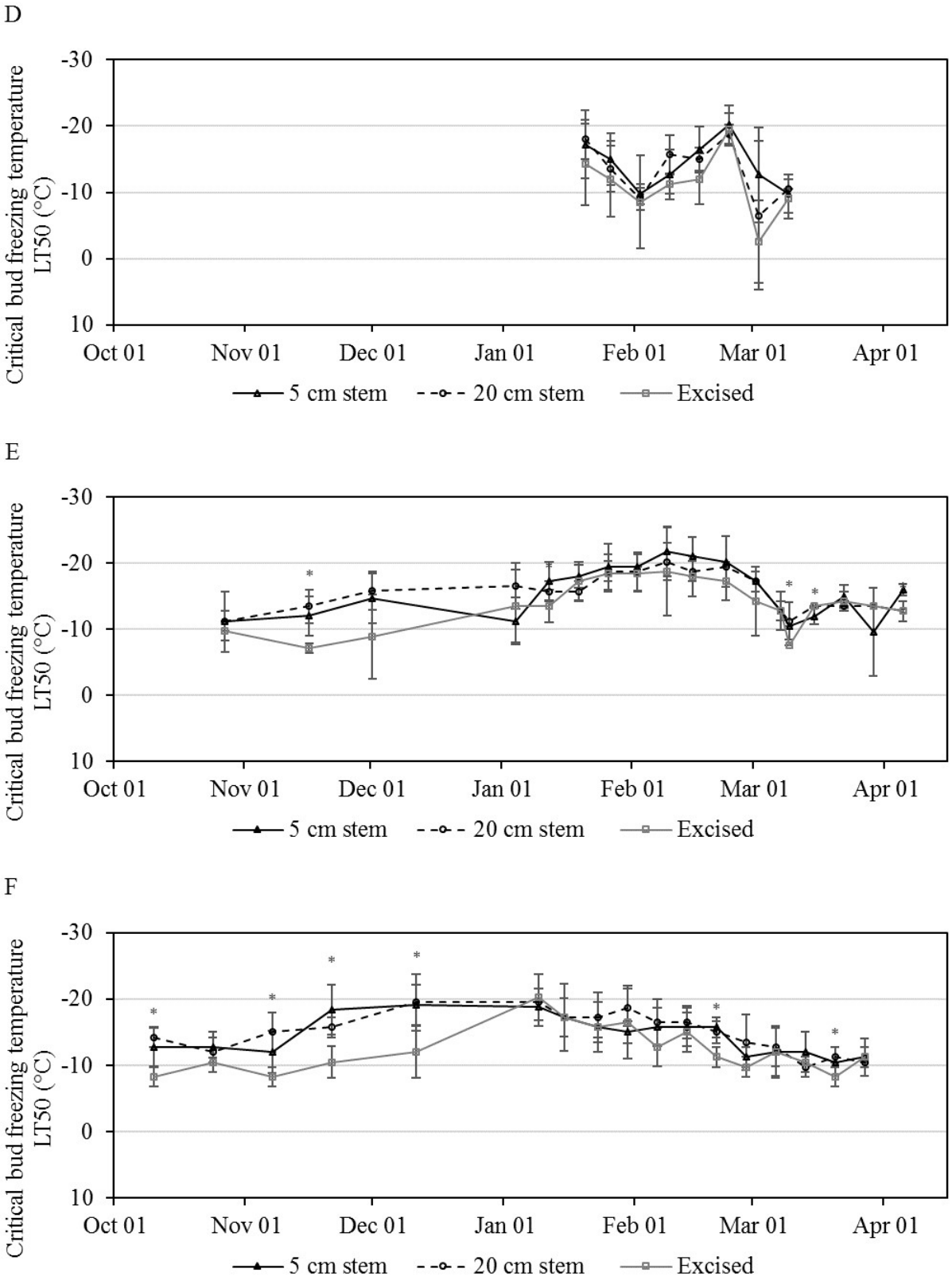
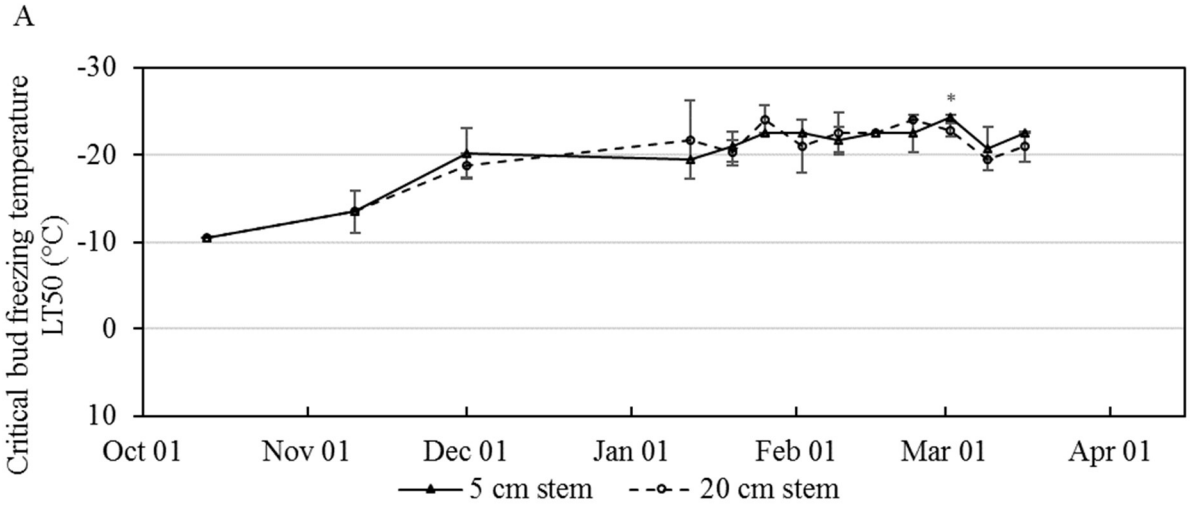
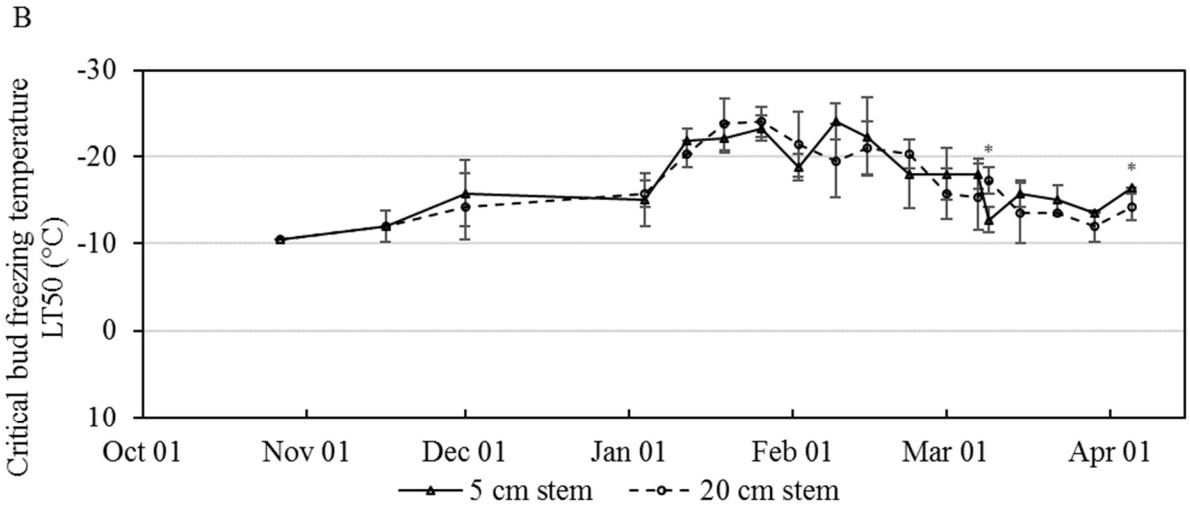
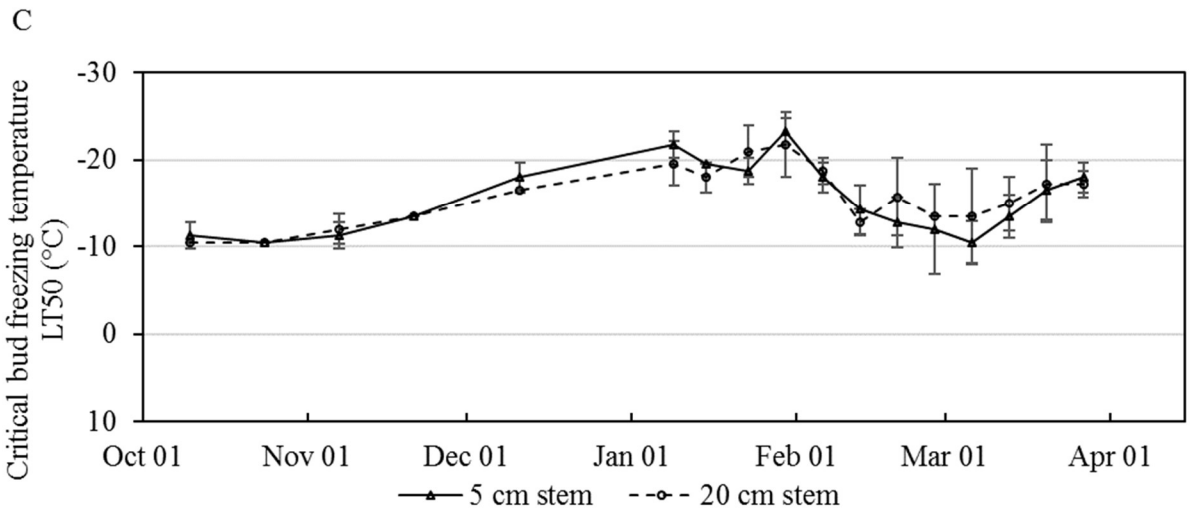


Fig. 2.2. Sampling types effect on critical bud freezing temperatures (LT_{50}) estimations for vegetative buds using the artificial freezing test. LT_{50} were calculated for vegetative

buds of ‘Elberta’ (A-C) and ‘Flavorich’ (D-F) for winter of 2014-2015 (A, D), 2015-2016 (B, E), and 2016-2017 (C, F). Statistical significant differences (P<0.05) determined by Student’s *t* test across different sampling types are indicated by asterisks. Bars indicate standard deviations.


A
Critical bud freezing temperature LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
5 cm stem
20 cm stem


B
Critical bud freezing temperature LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
5 cm stem
20 cm stem


C
Critical bud freezing temperature LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
5 cm stem
20 cm stem

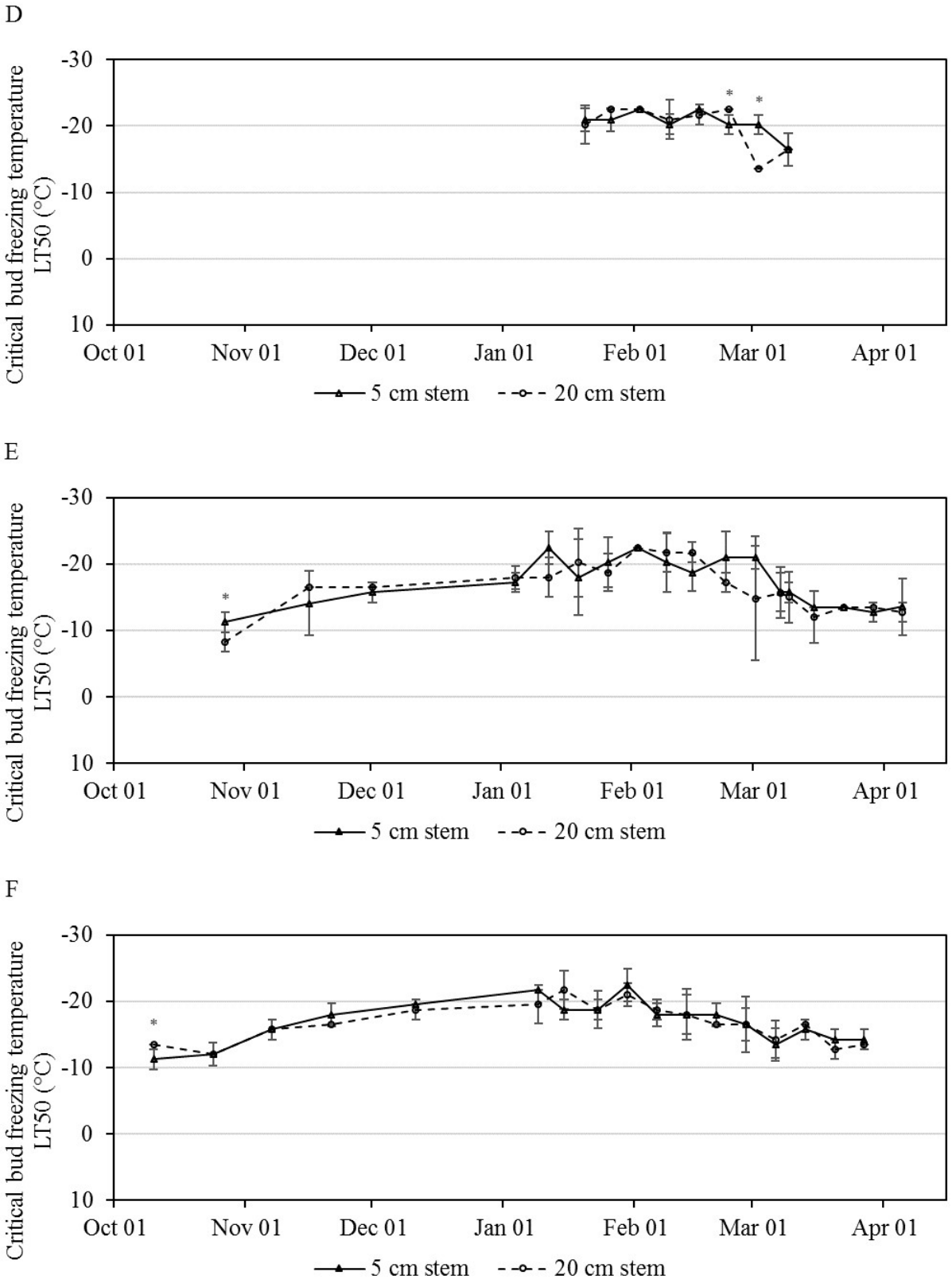
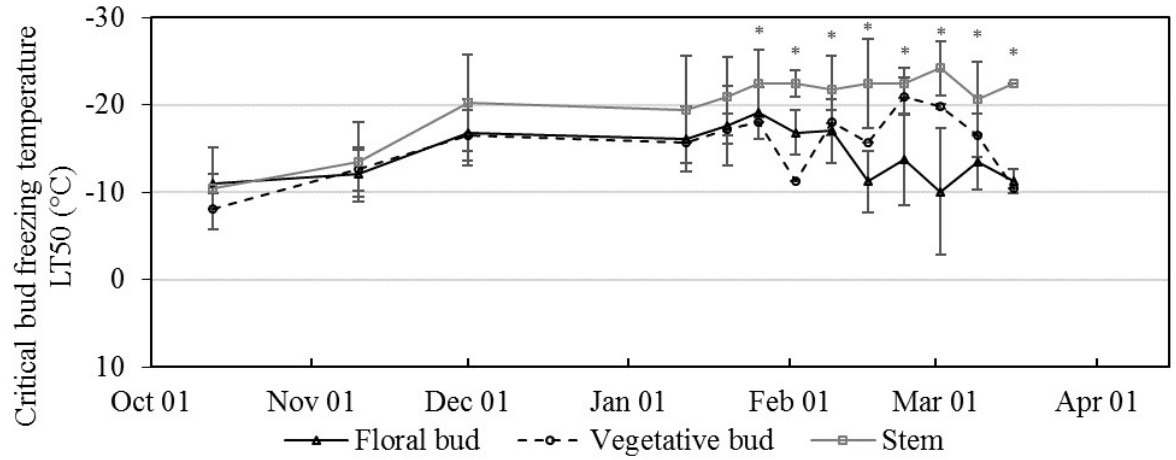


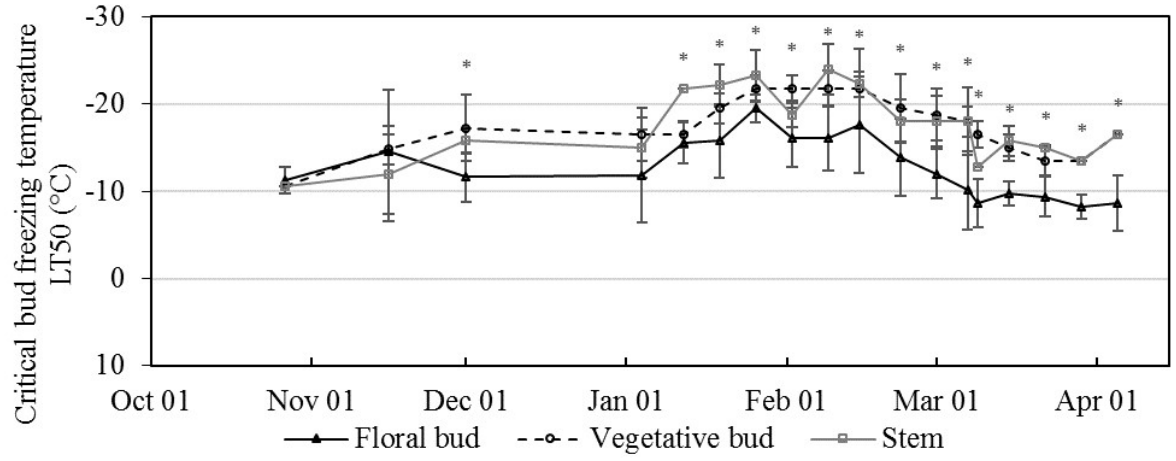
Fig. 2.3. Sampling types effect on critical freezing temperatures (LT_{50}) estimations for stems using the artificial freezing test. LT_{50} were calculated for stem tissue of 'Elberta'

(A-C) and ‘Flavorich’ (D-F) for winter of 2014-2015 (A, D), 2015-2016 (B, E), and 2016-2017 (C, F). Statistical significant differences (P<0.05) determined by Student’s t test for LT_{50} across different sampling types are indicated by asterisks. Bars indicate standard deviations.

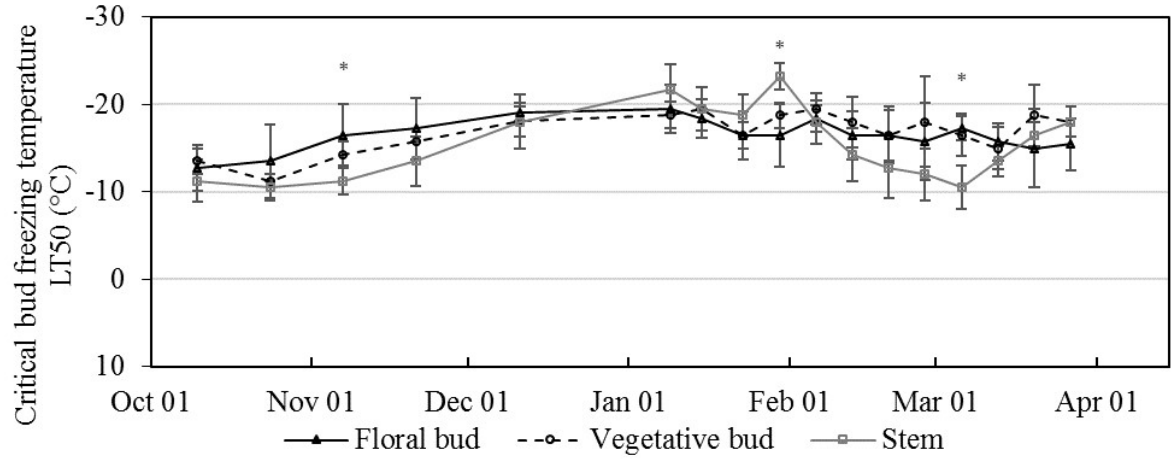
A


Critical bud freezing temperature LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud
Stem

B


Critical bud freezing temperature LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud
Stem

C


Critical bud freezing temperature LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud
Stem

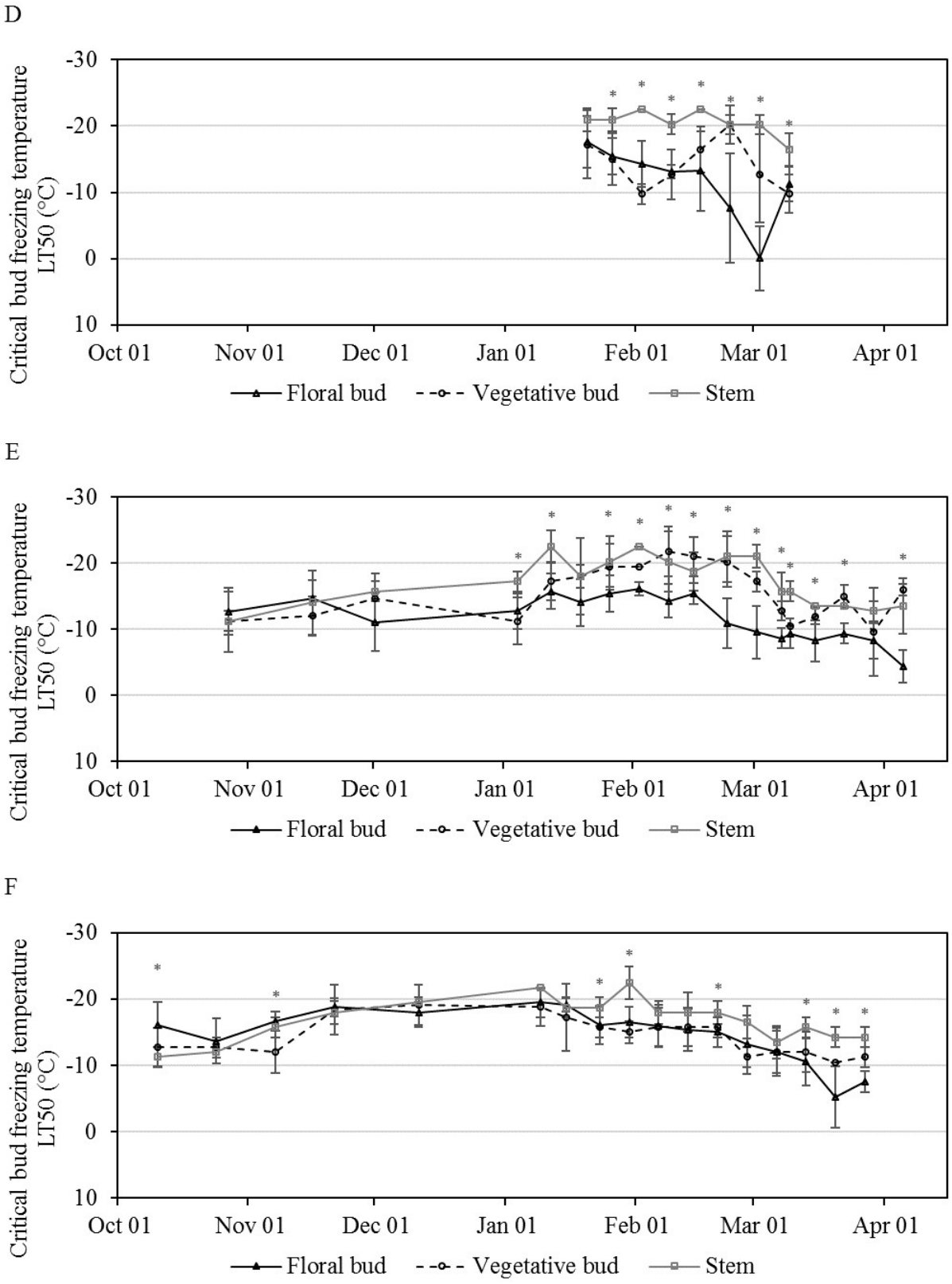
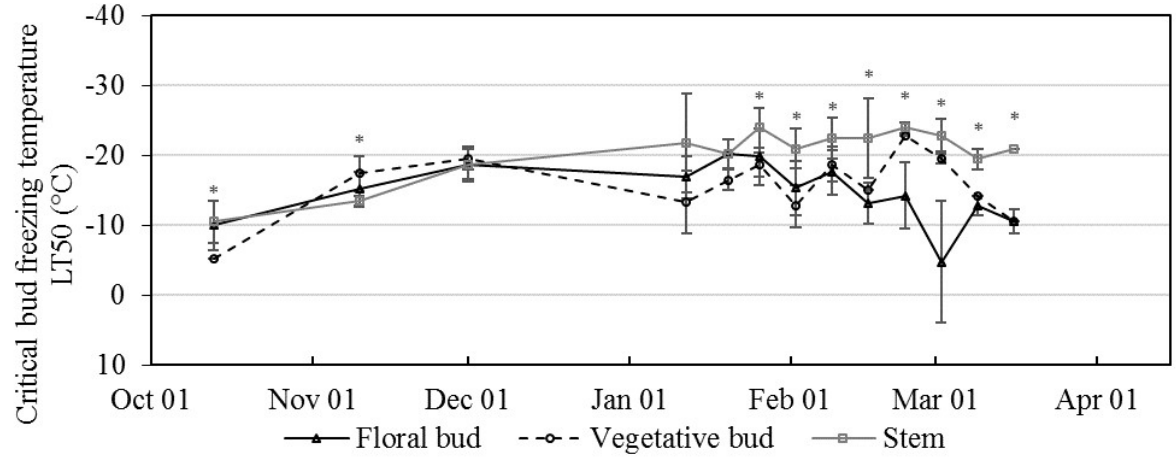


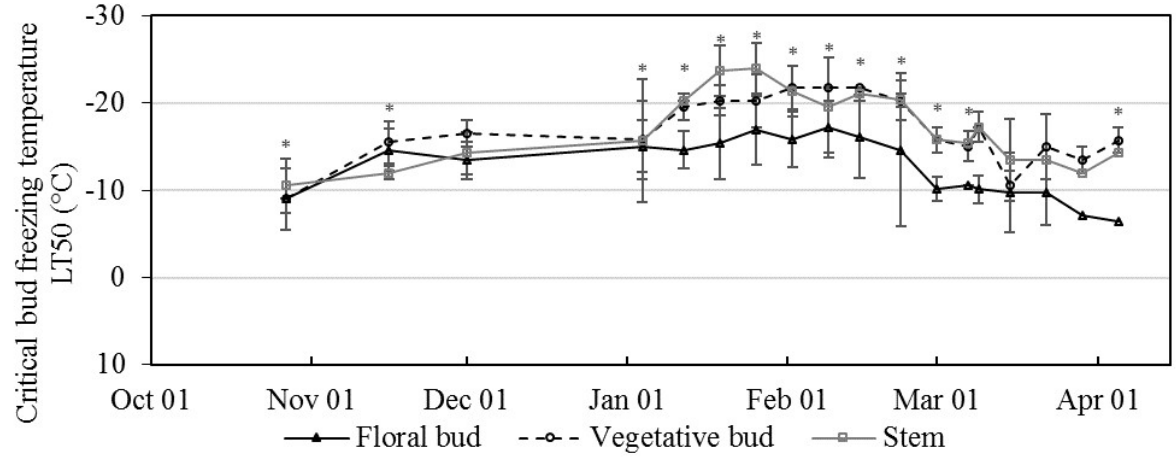
Fig. 2.4. Tissue types effect on critical freezing temperatures (LT_{50}) estimations for different tissues on 5 cm stem using the artificial freezing test. LT_{50} were calculated for

all tissue types of ‘Elberta’ (A-C) and ‘Flavorich’ (D-F) for winter of 2014-2015 (A, D), 2015-2016 (B, E), and 2016-2017 (C, F). Statistical significant differences (P<0.05) determined by Student’s t test for LT_{50} across different tissues are indicated by asterisks. Bars indicate standard deviations.

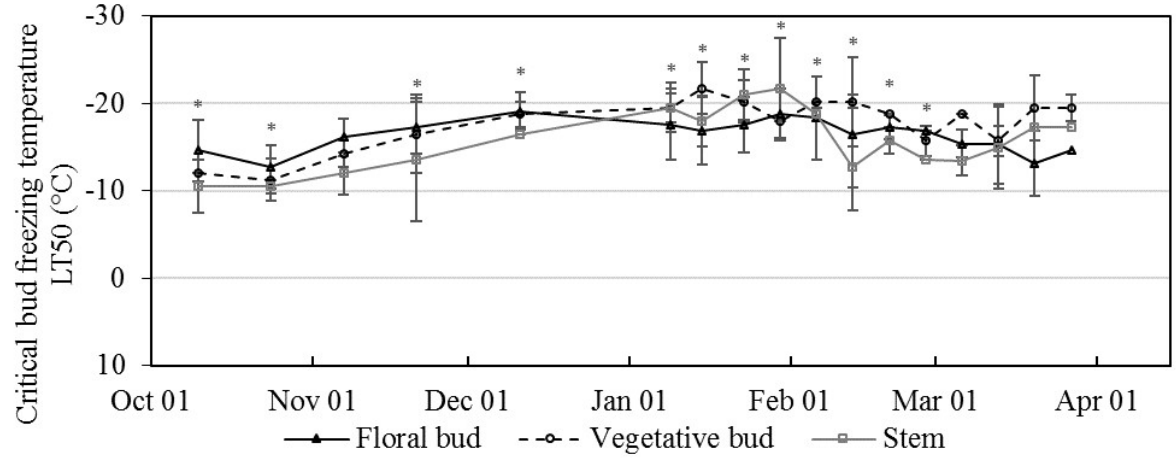
A


Critical bud freezing temperature
LT50 (°C)
-40
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud
Stem

B


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud
Stem

C


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud
Stem

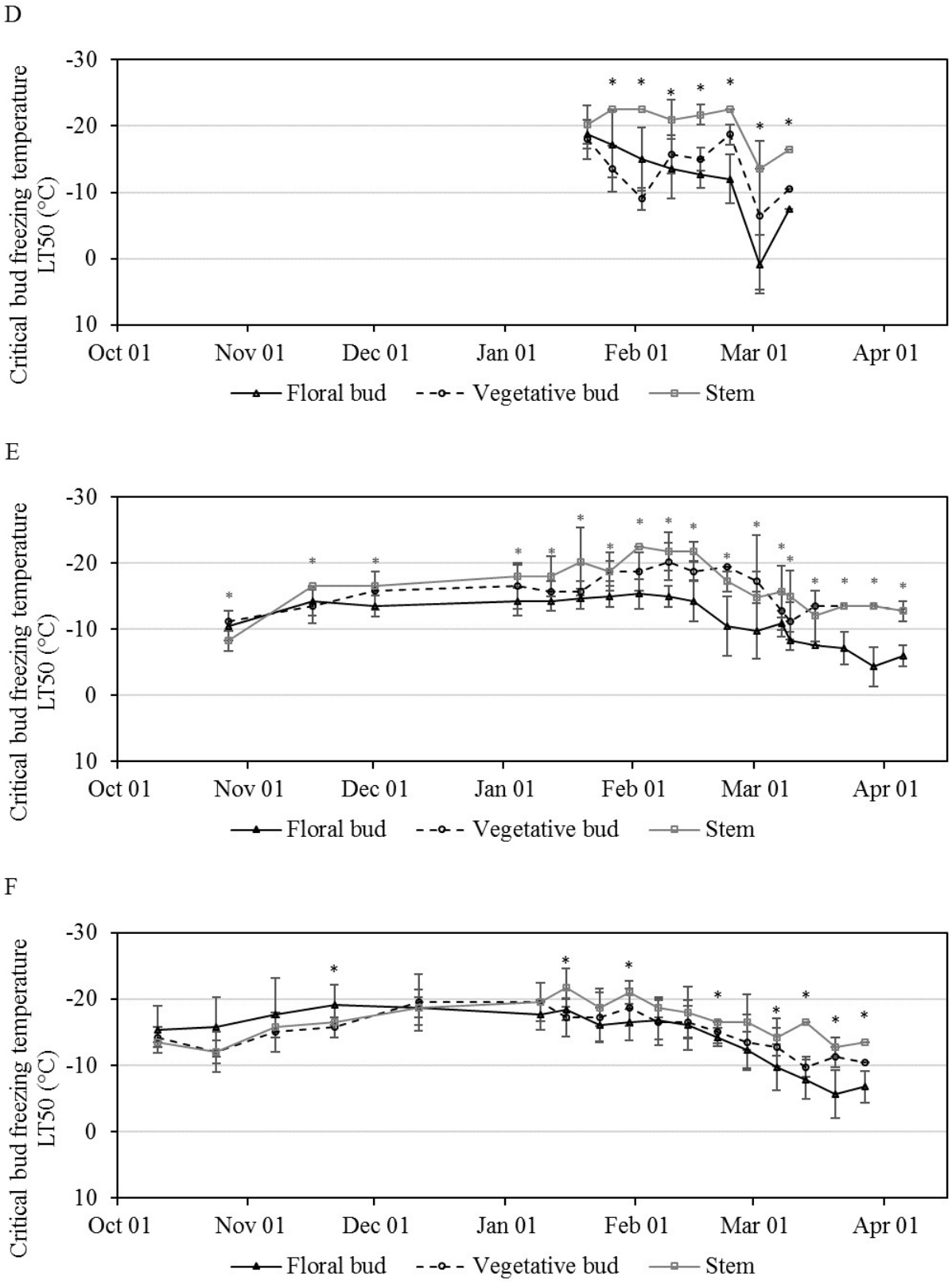
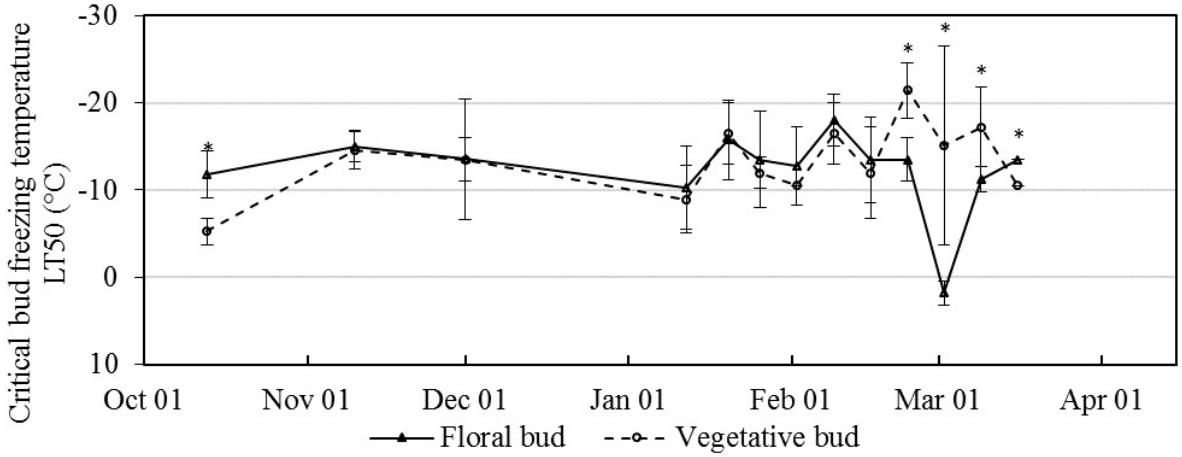


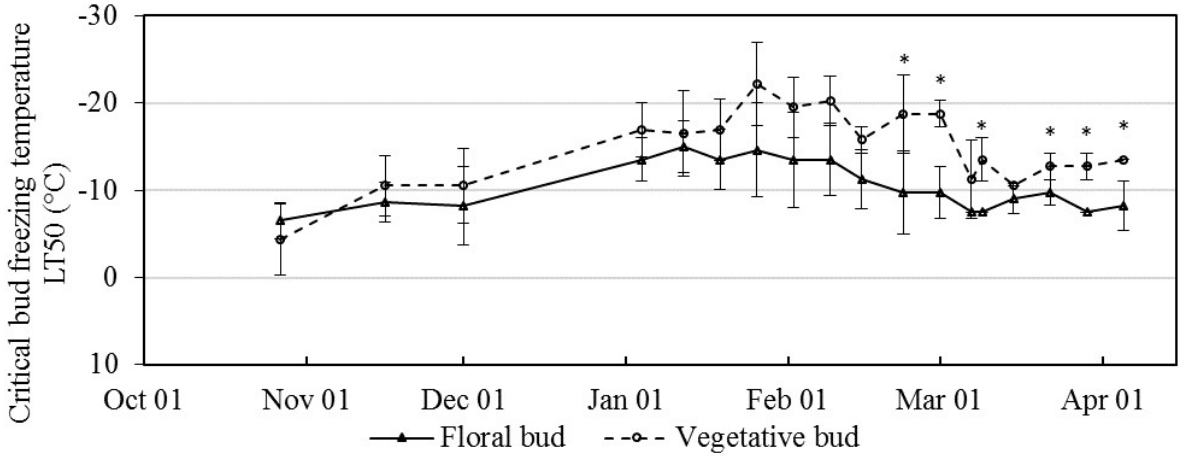
Fig. 2.5. Tissue types effect on critical freezing temperatures (LT_{50}) estimations for different tissues on 20 cm stem using the artificial freezing test. LT_{50} were calculated for

all tissue types of ‘Elberta’ (A-C) and ‘Flavorich’ (D-F) for winter of 2014-2015 (A, D), 2015-2016 (B, E), and 2016-2017 (C, F). Statistical significant differences (P<0.05) determined by Student’s t test for LT_{50} across different tissues are indicated by asterisks. Bars indicate standard deviations.

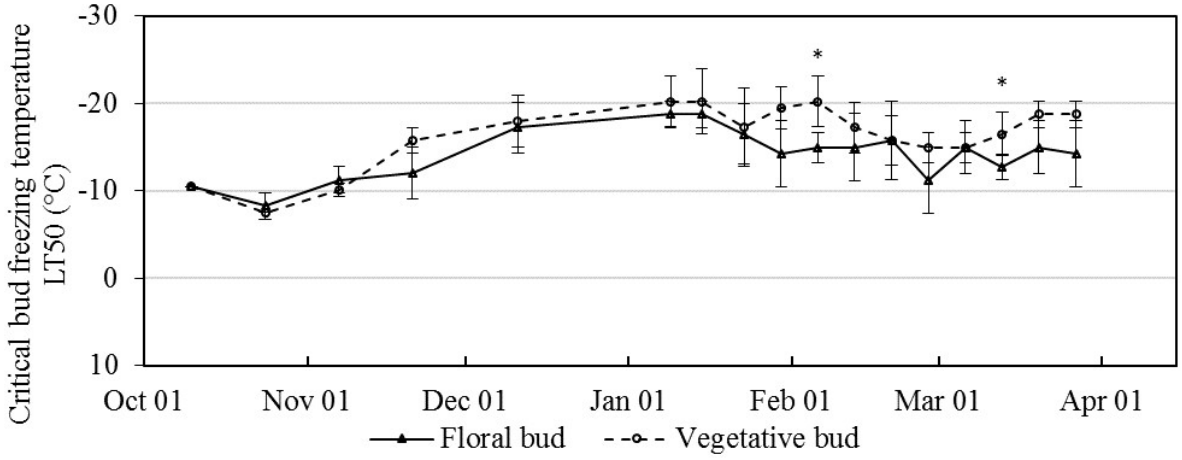
A


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud

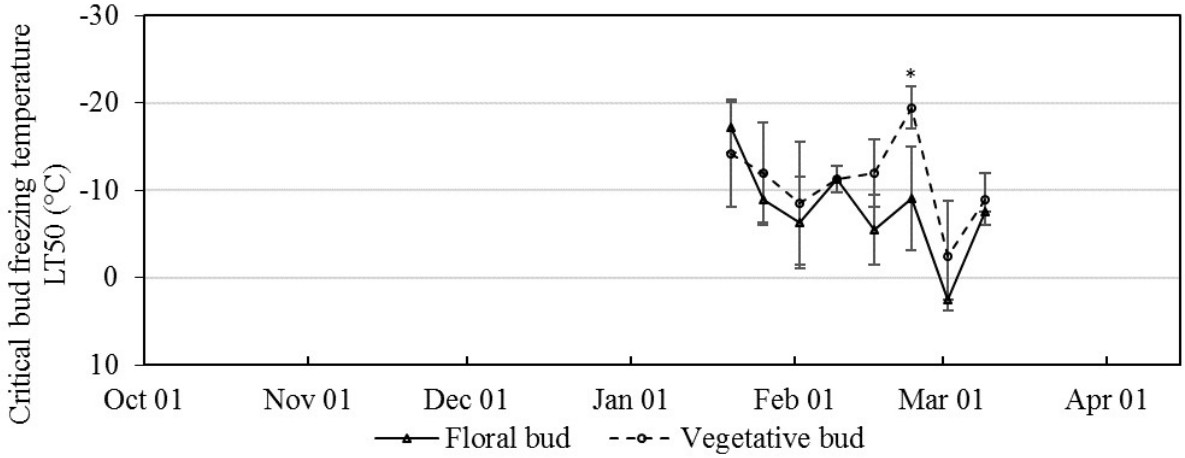
B


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud

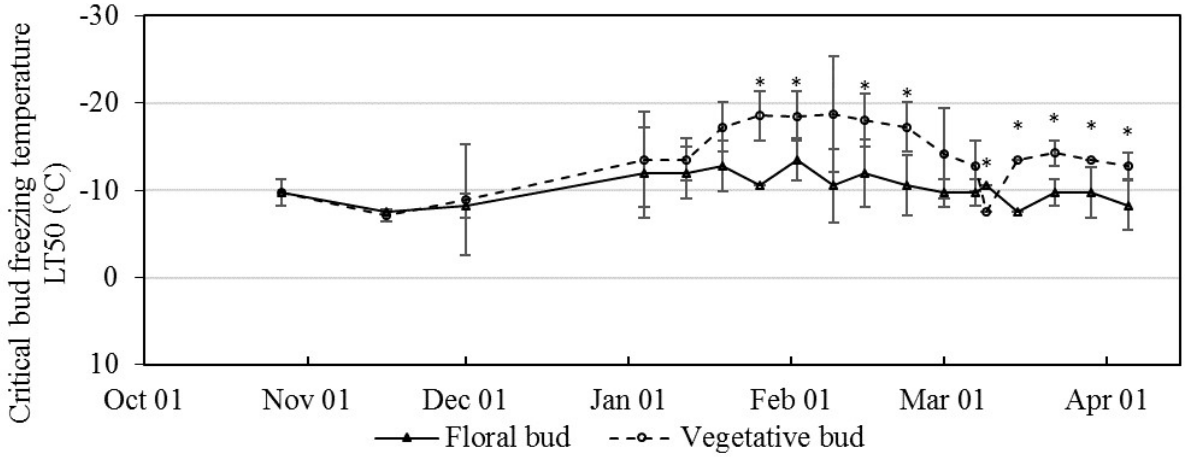
C


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud

D


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud

E


Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud

F

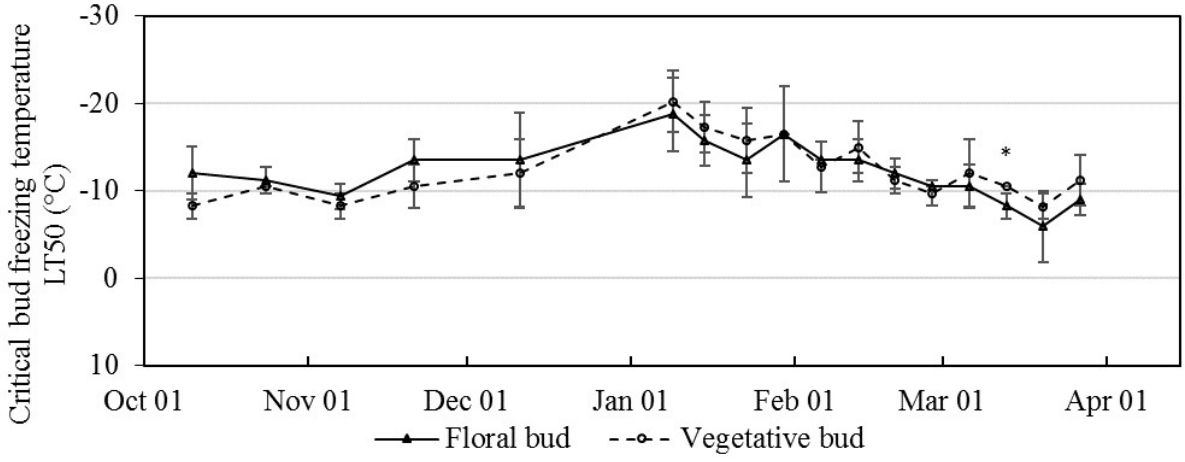
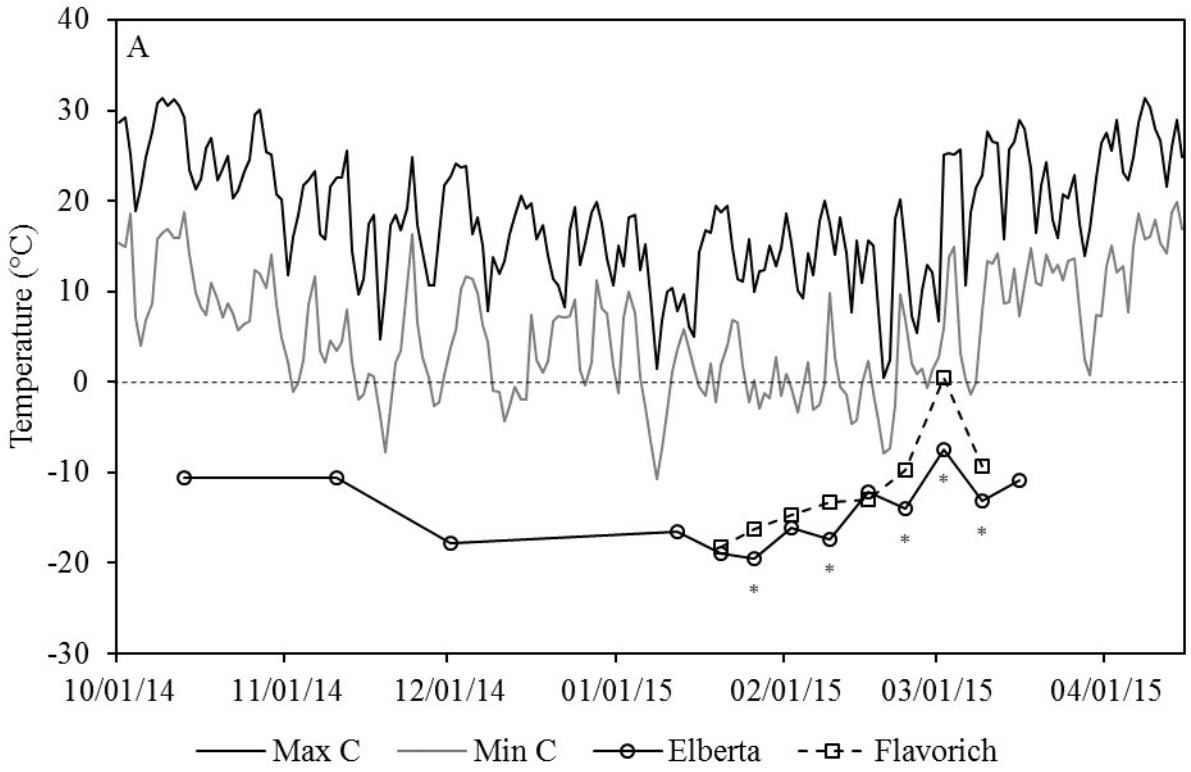
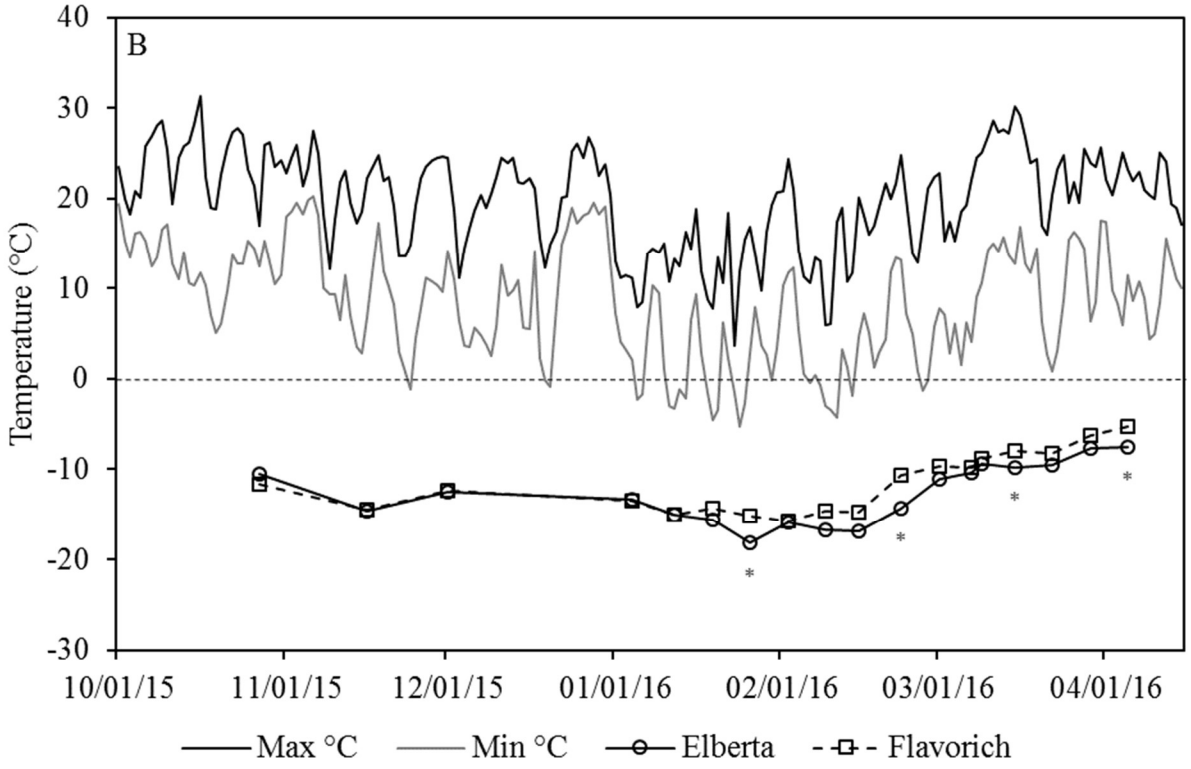

Critical bud freezing temperature
LT50 (°C)
-30
-20
-10
0
10
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
Floral bud
Vegetative bud

Fig. 2.6. Tissue types effect on critical bud temperature (LT_{50}) estimations for excised tissues. LT_{50} were calculated for floral and vegetative buds of 'Elberta' (A-C) and 'Flavorich' (D-F) for winter of 2014-2015 (A, D), 2015-2016 (B, E), and 2016-2017 (C, F). Statistically significant differences ($P<0.05$) determined by Student's *t* test between different tissues were observed, and are indicated by asterisks. Error bars indicate standard deviations.


A
Temperature (°C)
40
30
20
10
0
-10
-20
-30
10/01/14
11/01/14
12/01/14
01/01/15
02/01/15
03/01/15
04/01/15
Max C
Min C
Elberta
Flavorich


B
Temperature (°C)
40
30
20
10
0
-10
-20
-30
10/01/15
11/01/15
12/01/15
01/01/16
02/01/16
03/01/16
04/01/16
Max °C
Min °C
Elberta
Flavorich

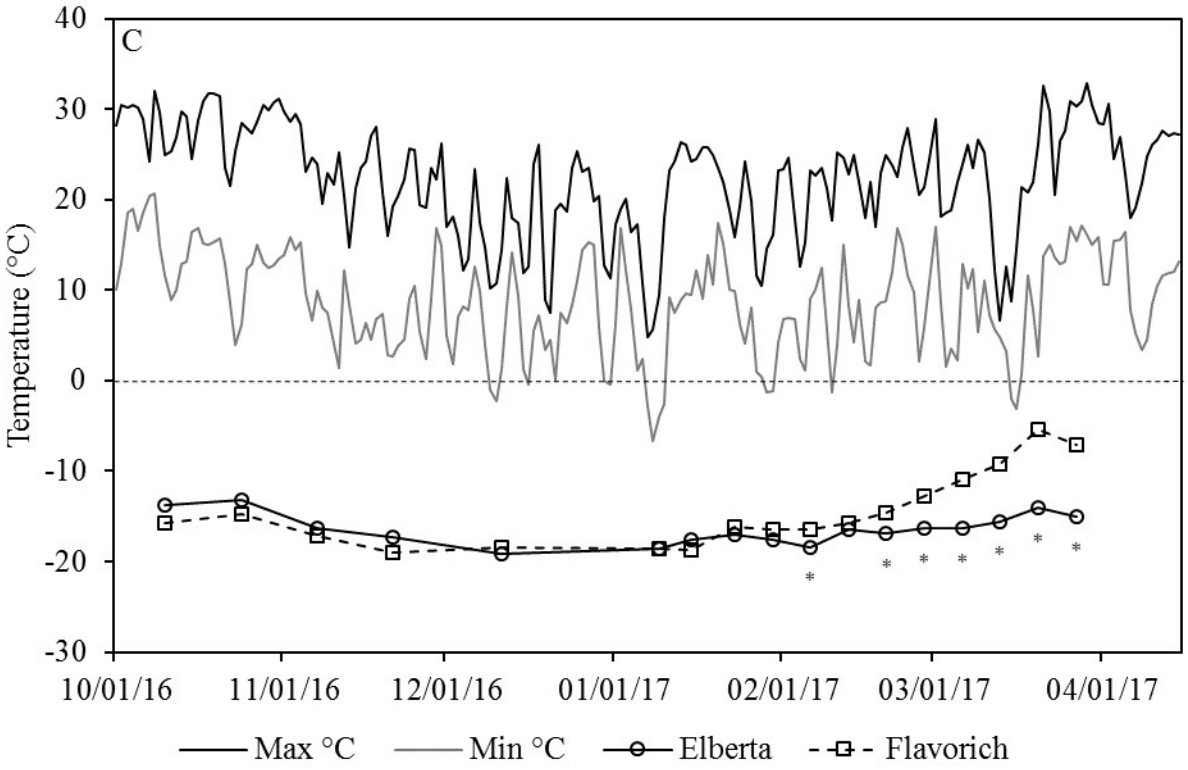


Fig. 2.7. Critical bud temperature values for 'Elberta' and ‘Flavorich’ floral buds (averages of 5 cm and 20 cm floral buds attached to stems) and daily temperature maximum (Max ˚C) and minimum (Min ˚C) for seasons of 2014-2015 (A), 2015-2016 (B), and 2016-2017 (C) in Fort Valley, GA, through winter and early spring. Floral buds attached to stems were used for this graph. Statistical significant differences (P<0.05) as determined by Student’s t test between different cultivars are reported and indicated by asterisks.

CHAPTER 3

COLD HARDINESS OF PEACH: IS DTA A GAME CHANGER?[1]

[1]Liu, J. et al. To be submitted to *HortScience*.

Abstract

Differential thermal analysis (DTA) has great potential as a quick and convenient cold hardiness determination method in plants. It measures freezing events inside of plant samples by detecting exotherm(s) produced when water changes from liquid to solid phase. DTA is highly sensitive to the experimental conditions and it has been reported to be ineffective among different fruit crops after acclimation of floral buds has occurred. The objective of this project was to establish DTA as a rapid and accurate method to measure peach floral bud cold hardiness as compared to the traditional standard artificial freezing test. The study consisted of modifying and optimizing a DTA protocol for peaches to produce meaningful cold hardiness results as compared against the standard test - the artificial freezing test. Floral buds of 'Elberta' and 'Flavorich' peach varieties were subjected to DTA and artificial freezing tests throughout the winters of 2015-2016 and 2016-2017. Lethal temperature that damaged 50% of floral buds (LT_{50}) was determined using the artificial freezing tests for both cultivars as a reference. Before deacclimation, two distinct exotherms were normally detected from floral bud DTA analyses. Low temperature exotherms (LTE) appeared at lower temperature and corresponded to freezing events that occurred inside of the bud and caused lethal damage to floral buds. High temperature exotherm (HTE) appeared at temperatures close to 0 °C, unlike LTEs, they are not directly associated with cold damage. After deacclimation, DTA tests yielded only few LTEs. However, incubation of floral buds at -2 °C overnight before the cooling process of DTA tests yielded an increase number of LTEs for both seasons in comparisons with samples directly run using DTA without incubation. Similarly, after deacclimation started, the temperature in which LTE occurred were

correlated (r=0.62 - 0.81) with LT_{50} when DTA samples were treated overnight at -2 °C. In our study, pre-treatment of floral buds at -2 °C overcame the inability of DTA to detect LTEs after deacclimation. This methodology produced consistent results in comparison with the artificial freezing test. DTA is a promising method to measure cold hardiness of peach plants. In the future, it would be important to better define the accuracy of DTA to predict cold hardiness of peach plants in field conditions.

Introduction

Peach, *Prunus Persica* (L.) Batsch, is an important fruit crop, ranking 4th worldwide after grape, apple, and pear. It is also the official fruit of the state of Georgia. In 2015, Georgia had 10,000 acres of bearing peach trees, producing a total of 40,600 tons of fruit with a farm gate value of almost 50 million dollars (USDA, 2016; Wolfe and Stubbs, 2016). In Georgia, a major threat to peach production is natural frost. Winter weather in Georgia is usually mild, and does not normally cause low temperature damage to peach plants. In spring, low temperature following warm period causes most cold damage and yield losses when the most vulnerable tissue - floral buds - deacclimate (lost cold hardiness). Peach plants start deacclimation and become susceptible to freezing temperatures at the end of February, but often spring frosts occur until late March. Late spring frosts result in crop losses as they affect early-, mid-, and late-season peach varieties. It is important to closely monitor the cold hardiness of peach plants through late winter and early spring, when fluctuating air temperatures coincide with the loss of floral bud cold hardiness. The use of different methods to measure peach cold hardiness could help researchers and peach growers to predict potential damage and yield losses that could be produced by an upcoming freeze. These methodologies will help to decide on the best course of action and protection strategy prior to the upcoming natural freeze.

Peach cold hardiness can be measured using the artificial freezing test. The artificial freezing test is commonly accepted as the standard test to estimate cold hardiness by applying artificial low temperature treatments to whole plant or plant tissues and evaluating directly cold damage. Cold injured tissue oxidizes and develops a yellow to brown color, thus the discoloration can be used as an indication for low temperature

damage. From the rate of injury, the freezing temperature that causes damage to 50% of the sample is known as lethal temperature 50 (LT_{50}) (Bigras and Colombo, 2013; Levitt, 1980; Stergios and Howell, 1973).

Peach floral buds can avoid freezing damage through supercooling (Quamme, 1974). Supercooling occurs when water inside of the acclimated floral bud primordia stays liquid even when temperature is below freezing. Cold acclimated floral buds prevent ice nucleation within its primordia, which is a prerequisite of freezing, by eliminating ice nucleating sites. In this way, winter floral buds avoid water freezing and freezing damage altogether. When temperature drop too fast and triggers freezing of supercooled water, the freezing is usually energetic and dramatic, and leads to plant tissue lethal damage. Water in the floral bud scales and flower axis do not exhibit supercooling and usually do not result in damage when frozen (Ashworth and Wisniewski, 1991; Levitt, 1980).

Differential thermal analysis (DTA) detects water freezing in plant tissues by identifying exotherms caused by freezing of water within a tissue. The exotherms are from released heat of fusion of water when it changes from liquid to solid phase. There are two distinct exotherm events in DTA analyses of an acclimated peach floral bud: 1) High temperature exotherm (HTE) occurs few degrees Celsius below 0 °C and is associated with water freezing in bud scales and the axis; and 2) Low temperature exotherm (LTE) occurs at a lower temperature and is associated with freezing of deep supercooled water and lethal damage to plant tissues. In this way, DTA can be used as well to measure cold hardiness of plants (Quamme et al., 1972).

DTA is an efficient and convenient way of assessing cold hardiness as compared with the artificial freezing test (Burr et al., 1990). Estimation of cold hardiness using DTA is available in few hours, in contrast of a week when using the artificial freezing test. Nevertheless, DTA does not directly indicate cold hardiness. It rather measures freezing temperature of supercooled water in tissues. The DTA profile can be affected by test conditions such as cooling rate, pre-treatment temperature, and sampling methods (Biermann et al., 1979; Flinn and Ashworth, 1994; Quamme, 1986). In addition, DTA was found to be ineffective to detect LTEs of blueberry floral buds when buds were attached to stems under a slow cooling rate mimicking natural freezing conditions (Flinn and Ashworth, 1994). Therefore, DTA is still argued as unreliable, although strong correlations between LTE from DTA analyses and LT_{50} values from artificial freezing test of peach floral buds have been previously reported (Proebsting Jr. and Sakai, 1979; Quamme, 1974; Quamme et al., 1975). Understanding the DTA analyses in peach and developing a more reliable DTA protocol that can be deploy in few hours is an important priority to manage climatic conditions that are detrimental to agricultural production. DTA will provide peach growers with up-to-date information of their peach varieties' cold hardiness. The objective of this study is to develop DTA as a useful and accurate method to measure cold hardiness of peach floral buds, by building correlations between DTA and artificial freezing test and calibrating DTA against artificial freezing test.

Materials and Methods

Plant material

Twigs were randomly selected from 10-year old 'Elberta' and 'Flavorich' peach trees grafted on 'Guardian' rootstocks growing in a commercial orchard at Fort Valley, GA maintained following commercial guidelines (Horton et al., 2010). Samples were collected monthly from November 2015 to December 2015, bi-weekly from October 2016 to December 2016, and weekly from January to March of both 2016 and 2017. Approximately, 60 stems per variety were collected at each collection date and transported to the University of Georgia Griffin Campus, Griffin, GA in a cooler with ice. Samples were then kept in a refrigerator at 4°C before being processed. Floral buds were excised for DTA tests. Stems were also trimmed into either stem sections of 5 cm or 20 cm with flower buds attached and were used for artificial freezing test as the reference test.

Differential thermal analysis tests

DTA were performed using thermoelectric modules (TEM) placed in a temperature-controlled freezing chamber (Temperature and humidity chamber PR-3FPH, Tabai ESPEC, Japan). Each TEM contains 240 thermocouples to detect the release of heat produced from water freezing. Four floral buds of the same cultivar were placed per TEM and covered with an aluminum lid to keep buds in constant contact with the TEM and to reduce the noise produced by the constant air cycling inside the freezer. Twelve TEMs were used with buds of the same cultivar per DTA test per collection date (six TEMs were used from October to December 2015). In addition, two DTA tests were

conducted with different pre-treatment cooling schemes per collection date. The first DTA test, denoted hereafter as "regular DTA", used a cooling scheme that dropped temperature quickly to -2˚C, and then decreased temperature at a constant rate of 4 ˚C·h^{-1} until it reached -27 ˚C. For the second DTA test, denoted hereafter as "pre-treated DTA", samples were first incubated at -2 °C overnight as a pre-treatment, then were cooled at a rate of -4 ˚C·h^{-1} until reaching -27 ˚C. The regular DTA was the standard method used throughout most of the experiments, however, the pre-treated DTA test was created to improve exotherm identification in samples during deacclimation. The pre-treated DTA started on January 26, 2016, and continued after that date. Three temperature probes were employed to record the temperature inside of the freezing chamber simultaneously when DTA tests were running. Temperatures when freezing events happened can therefore be traced. A Campbell Scientific data logger was connected to the TEMs and temperature probes to record the voltage signal and temperature every minute. The data were then plotted against time using SigmaPlot 13.0 software (San Jose, CA, USA) to identify LTE and HTE. A connection error created issues with the DTA test of October 2015 and data failed to be recorded.

Artificial freezing test

Artificial freezing tests were used as reference to detect tissue damage associated with freezing. Stems of both 5 cm and 20 cm with floral buds were wrapped in a damp tissue paper at the basal end to prevent wilting. A temperature treatment gradient of ten temperatures was used for this test, which were 4 °C (control), -3 °C, -6 °C, -9 °C, -12 °C, -15 °C, -18 °C, -21 °C, -24 °C and -27 °C. At each temperature, four replicates per

sample type (5 cm stem and 20 cm stem) per cultivar were assigned and sealed in the same plastic bag corresponding to a freezing temperature treatment. The same temperature-controlled freezing chamber was used as DTA test. Samples in bags were kept in the freezing chamber at a constant -2 °C overnight. In the morning, the freezing chamber then was set to a decreasing rate of -4 °C·h^{-1} starting at -2 °C until reaching -27 °C. Bags corresponding to a temperature treatment were rapidly removed from the freezing chamber when the temperature inside the chamber reached their corresponding treatment temperature. Treatment temperatures were measured by three temperature probes placed in bags at different locations in the chamber. After the freezing treatments were done, samples were kept in a refrigerator for a week at 4 °C to recover and allow discoloration to develop. Floral buds were then evaluated for freezing damage by dissecting them longitudinally with a razor blade. Samples that showed discoloration and oxidation were scored as death. Samples with green and healthy structures were scored as alive. A total of eight floral buds were evaluated for each temperature treatment per cultivar. In a previous study (Liu et al., unpublished data), sampling types (floral buds attached to 5 cm stem vs. floral buds attached to 20 cm stems) were shown to be not significantly different from each other and not to affect cold hardiness estimations. Therefore, critical bud temperature calculations were based on an average of the 16 replicates for the two sampling types of floral buds attached to stems (5 cm stem and 20 cm stems).

Data analysis

Temperatures corresponding to each exotherm per TEM were identified from the DTA profiles. The distribution of temperatures corresponding to each exotherm for each cultivar were evaluated using JMP Pro 13 software (SAS Institute Inc., Cary, NC, USA). Usually, the distribution of exotherm temperatures fitted a Normal 2 Mixture distributions. The first normal distribution corresponded to exotherms at higher temperatures and were considered HTEs. The second normal distribution corresponded to exotherms at lower temperatures and were considered LTEs (Figs. 3.1 and 3.2). Means of the Normal 2 Mixture distributions corresponding to HTE or LTE temperatures were used for further analyses. In few cases when using the pre-treated DTA, the distribution of exotherm temperatures fitted one normal distribution with all exotherms identified as LTEs. Examples of fitted distribution are shown in Fig. 3.2.

Critical bud temperatures (LT_{50}) were calculated from the results of visual evaluation from the artificial freezing tests. A nominal logistic model was fitted using the visual rating of buds to determine LT_{50} with JMP Pro 13. Pearson's correlations between critical bud temperature from artificial freezing test and LTEs temperatures from DTA tests were performed. In addition, regression analyses with critical bud temperatures as the dependent variable and the LTEs from DTA as the independent variables were carried out with JMP Pro 13.

Results

Overall result and LTE detection. Exotherms were detected in floral buds of 'Elberta' and 'Flavorich' peach varieties (Fig. 3.1). Apart from occurring at a lower temperature, LTE

peaks in DTA profiles tended to be sharper and narrower than HTE peaks. In the 2015-2016 season, HTEs could always be detected. LTEs, in the other hand, were identified by regular DTA only before February 23, 2016 (Table 3.1 and Table 3.2). For both varieties in the 2015-2016 season, the lowest LTE temperatures using the regular DTA were observed on January 12, 2016, with LTE temperature of 'Elberta' being -21.1 °C and LTE of 'Flavorich' being -16.2 °C. As floral buds developed and progressed towards deacclimation, LTE peaks in DTA profiles grew taller and shifted to higher temperatures. After February 23, 2016, exotherm peaks in the profile of regular DTA were wide and overlapping with each other (Fig. 3.1C and 3.1G), and LTEs were unable to be recognized due to loss of the peach floral buds' supercooling capability (Ashworth, 1984). DTA tests with -2 °C pre-treatment started on January 26, 2016. LTEs of 'Elberta' were stable around -18 °C from the start date of study to February 23, 2016, suggesting full acclimation. For 'Flavorich' floral buds, LTE temperature from pre-treated DTA showed more fluctuation (Fig. 3.3C). Afterward, LTE peaks from pre-treated DTA tests shifted towards warmer temperatures and became fewer in number, but were still able to be distinguished by their narrow and shaper shapes within the DTA profiles towards the end of March (Fig. 3.1D and 3.1H, and Table 3.1).

For the 2016-2017 season, the southeastern U.S. was characterized by an extreme warm winter. The chilling requirement (number of hours below 7.2 °C and above 0 °C that plants need to experience in order to break out of dormancy) of 'Elberta' and 'Flavorich' are 850 and 650 chilling hours (USDA, 2017), respectively. In our experiment, at the last sampling date of March 27, 2017, Fort Valley, GA had only accumulated 633 chilling hours compared to 1100-1300 chilling hours accumulated in

average (Georgia Automated Environmental Monitoring Network, 2017). The lack of chill in the 2016-2017 season resulted in failure of dormancy break of 'Elberta' floral buds and a slow and non-uniform bud break of 'Flavorich' floral buds. Until the end of this study (end of March), only few floral buds of 'Elberta' had broken in the orchard. The difference of responses to the natural accumulation of chill for the 2016-2017 season for 'Elberta' and 'Flavorich' floral buds were also reflected in their DTA profiles (Fig. 3.3B and 3.3D).

No LTEs were detected for any DTA test at the beginning of the test periods of the 2016-2017 season, except for regular DTA on October 10, 2016 (Table 3.1 and Table 3.2). After December 11, 2016, LTEs could be observed in all DTA test, except for regular DTA on January 9, 2017. For 'Elberta' floral buds, the lowest LTE estimated by regular DTA and pre-treated DTA were -18.7 °C on February 6, 2017 and -20.1 °C on January 15, 2017, respectively (Fig. 3.3B and Table 3.1). Afterward, both pre-treated DTA and regular DTA were able to detected LTEs until early spring (Fig. 3.3B and Table 3.1). LTEs of 'Elberta' floral buds obtained by both regular DTA and pre-treated DTA did not reflect a clear acclimation and deacclimation pattern (Fig. 3.3B). Even on the last sampling date, LTEs of 'Elberta' floral buds were -16.8 °C and -17.2 °C for regular DTA and pre-treated DTA, which were close to the lowest LTE temperature (Table 3.1). For 'Flavorich', the acclimation process was not captured by the DTA tests either, since DTA data was only available from December 11, 2017 (Fig. 3.3D). Although, LTEs measured by regular DTA test for 'Flavorich' were -17.0 °C on December 11, 2016, which is a large drop from -9.2 °C of the last available data on October 10, 2016 (Table 3.2). LTE temperatures for regular DTA of 'Flavorich' floral buds were around -17 °C from

December 11, 2016 to February 20, 2017, then started to increase afterward and reached to -14.6 °C at the last sampling date on March 27, 2017 (Fig. 3.3D and Table 3.2). LTE of regular DTA were always lower when compared with LTEs obtained during the 2015-2016 season tests corresponding to the same period of analysis during the 2016-2017 season. Pre-treated DTA obtained the lowest LTE temperatures of -21.6 °C on December 11, 2016 for 'Flavorich' floral buds. LTE temperature then increased slowly until reaching -14.1 °C on the last sampling date. Similarly, LTEs of 'Flavorich' of pre-treated DTA in the season of 2015-2016 test had more variation and fluctuation and yielded higher temperature LTEs than the 2016-2017 test of the same period (For example, 'Flavorich' $LTE_{pre\text{-}treated}$ of -7.7 °C on March 9, 2016 and -10.2 °C on March 15, 2016 vs. $LTE_{pre\text{-}treated}$ of -16.0 °C on March 6, 2017 and -14.4 °C on March 13, 2017) (Table 3.2).

The number of LTE peaks detected for both DTA tests (regular and pre-treated) for each date were counted (Table 3.1 and Table 3.2). The number of LTE peaks identified by using the pre-treated DTA were usually more than regular DTA, especially after deacclimation.

Water content. Seasonal changes of water content of floral buds from both cultivars were recorded (Fig. 3.4). Floral buds water content reminded stable during acclimation, and increased dramatically after floral bud deacclimation, which is clear for both cultivars in the 2015-2016 season (Fig. 3.4A) and 'Flavorich' floral buds in the 2016-2017 season (Fig. 3.4B). In the first season, deacclimation of both cultivars started on February 23, 2016; in the second season, 'Flavorich' floral buds started deacclimating on January 23, 2017. Floral buds of 'Elberta' did not deacclimate in the second season, and water

content did not show the sharp increase as 'Flavorich' floral buds did (Fig. 3.4B). Water content of floral buds correlated well with LT_{50} after deacclimation across two winters for both cultivars, except for 'Elberta' in the 2016-2017 test in which buds failed to deacclimate. The correlations were high, with r=0.84 for 'Elberta' in the 2015-2016 season, r=0.94 and r=0.95 for 'Flavorich' in the 2015-2016 and 2016-2017 seasons, respectively. During deacclimation, water content also correlated well with the LTEs observed by the pre-treated DTA, r= 0.87 for 'Elberta' in the 2015-2016 season, r= 0.66 and 0.91 for 'Flavorich' in the 2015-2016 and 2016-2017 seasons, respectively.

Effect of pre-treatment. In the 2015-2016 test, LTEs from DTA tests with the -2 °C pre-treatment were reported at lower temperatures than LTEs measured by regular DTA for the all dates evaluated when data from both DTA tests were available (Fig. 3.3A and 3.3C). Deacclimation started on February 23, 2016 for both cultivars, according to the artificial freezing test, which coincided with a sharp decline of the number of LTE peaks being detected using regular DTA (Table 3.1 and Table 3.2). Afterward, regular DTAs only yielded one or two HTE peak(s) around -5 °C (Fig. 3.1C and 3.1G). Pre-treated DTA tests, on the other hand, still had distinct peaks at lower temperatures (Fig. 3.1D and 3.1H).

LTE temperature of regular DTA and pre-treated DTA were close in the 2016-2017 test (Fig. 3.3B and 3.3D). LTE temperatures of 'Elberta' using the regular DTA were generally higher than those of the pre-treated DTA except in two dates (within 0.3 °C of difference) (Fig. 3.3B). The results were consistent with the test of the 2015-2016 season, when LTE temperature from pre-treated DTA tended to be lowest as well for

'Elberta' (Fig. 3.3A). LTE temperatures of 'Flavorich' floral buds for the two DTA tests in the 2016-2017 season did not show a clear pattern (Fig. 3D).

Correlations between LTE and LT_{50} were also examined to evaluate the credibility of utilizing the DTA test to predict the critical bud temperature of peaches. Overall, for 'Elberta', LTE temperatures from both DTA tests had a strong correlation with LT_{50}, with r=0.84 for regular DTA and r=0.88 for pre-treated DTA. As for 'Flavorich' floral buds, LTE temperatures correlated better with LT_{50} if samples were treated with -2 °C overnight vs. normal DTA, with a correlation coefficient of 0.76 vs. 0.44, respectively.

A closer examination was made on data collected after deacclimation. Acclimation and deacclimation dates of floral buds were defined by the artificial freezing test results. After deacclimation, pre-treated DTA were not only able to detect more LTE peaks, but also correlated better with the artificial freezing tests than regular DTA results. In the 2015-2016 season, peach floral buds' deacclimation initiated on February 23, 2016 for both varieties. After deacclimation, regular DTA failed to capture LTEs, while LTEs reported by pre-treated DTA were shown to strongly correlated with LT_{50}. The correlation coefficient between LTE temperatures and LT_{50} was 0.90 for 'Elberta' and 0.80 for 'Flavorich' after deacclimation. During the 2016-2017 season, however, the artificial freezing test did not show clear deacclimation process as 'Elberta' floral buds did not progress beyond bud swell, which is the first growth stage of peach after buds break dormancy. Overall the correlation coefficient between LTEs and LT_{50} were 0.66 for regular DTA and 0.68 for pre-treated DTA in the 2016-2017 season. Floral buds of 'Flavorich', on the other hands, started deacclimation around January 23, 2017. After the

date, LTE of pre-treated DTA were strongly correlated with LT_{50} at r=0.94, compared to r=0.65 between LTE from regular DTA and LT_{50} from the same period after deacclimation.

Linear regressions were tested to compare the reliability of regular DTA and pre-treated DTA to predict cold hardiness. The regression equation to predict LT_{50} from $LTE_{regular}$ for 'Elberta' floral buds was $LT_{50} = (0.29 \times LTE_{regular}) - 11.30$ [$R^2 = 0.23$], and $LT_{50} = (0.26 \times LTE_{regular}) - 9.96$ [$R^2 = 0.03$] for 'Flavorich' floral buds. The regression equation to predict LT_{50} from $LTE_{pre\text{-}treated}$ for 'Elberta' floral buds was $LT_{50} = (0.87 \times LTE_{pre\text{-}treated}) - 0.23$ [$R^2 = 0.74$], and $LT_{50} = (0.97 \times LTE_{pre\text{-}treated}) + 2.68$ [$R^2 = 0.60$] for 'Flavorich' floral buds. The coefficient of determination of regression from $LTE_{regular}$ was lower than $LTE_{pre\text{-}treated}$.

Discussion

In this study, we explored different methods to estimate cold hardiness of peach in the Southeast region of U.S. The artificial freezing test and the LT_{50} temperature have been long used to test and express freezing resistance of plants (Levitt, 1980). Differential thermal analysis is a relatively new method known by its objectivity and rapidity (Burr et al., 1990) and having great potential for cold hardiness measurement. However, DTA's credibility to measure cold hardiness is still open to dispute (Flinn and Ashworth, 1994). DTA is highly sensitive to experimental conditions such as cooling rate temperature treatment, bud excision, pre-treatment temperatures, etc. (Andrews and Proebsting Jr., 1987; Andrews et al., 1983; Biermann et al., 1979).

The cooling rate for DTA has been recommended to be close to freezing temperatures occurring in nature (1-2 °C·h^{-1}) (Flinn and Ashworth, 1994). Proebsting Jr. and Sakai (1979) also suggested using a cooling rate "that will not affect the result". Yet experimental conditions that resemble nature (low cooling rate of 2 °C·h^{-1} and floral buds attached to stem) failed to detect LTEs using DTA in the case of blueberry floral buds (Flinn and Ashworth, 1994). Levitt (1980) suggested a standardized cooling rate for artificial freezing test, which applies for DTA as well. In our study, we tested two cooling schemes. Regular DTA test started with a temperature of -2 °C and then dropped air temperature to -27 °C with a cooling rate of 4 °C·h^{-1}. The other DTA test, pre-treated DTA, consisted of incubating the sample at -2 °C overnight and then following the same cooling scheme as regular DTA. In our study, we observed that pre-treated DTA was shown to perform better than regular DTA especially after floral bud deacclimation. It detected LTEs when regular DTA failed in the 2015-2016 season, and captured more LTE peaks than regular DTA in the 2016-2017 season (Fig. 3.1, Table 3.1 and Table 3.2). Pre-treated DTAs were also shown to correlate better with the artificial freezing test than regular DTA.

Low temperature at -2 °C overnight allowed water to relocate from primordia cells to apoplastic spaces, bud scales and axis, breaking continuity of the water within the bud which serves as ice propagation path (Quamme 1983, 1986). Incubating samples at -2 °C blocked ice nucleation outside of primordia and promoted supercooling without inducing additional low temperature damage. Therefore, the pre-treatment separated merging peaks in the DTA profile of deacclimated buds and facilitated detection of LTEs. Water relocation also reduced water content within bud primordia. Floral buds with low

water content have lower chance to trigger ice nucleation, thus freeze at a lower temperature. In our study, we noted that LTEs of pre-treated DTA tended to occur at a lower temperature than LTEs from regular DTA (Table 3.1 and Table 3.2). Similar water movement was observed in peach floral buds after acclimation at -10 °C for 10 d by Quamme (1983). LTEs of those buds appeared at lower colder temperature than buds treated with 0 °C (Quamme, 1983).

The pattern of LTE temperatures of pre-treated DTA were lower than that of regular DTA. This pattern clearly holds true for ‘Elberta’ for both years and for 'Flavorich' for season one, but not so for ‘Flavorich’ in the 2016-2017 season (Fig. 3.3D). Xylem continuity between floral bud primordia and bud axis can play an important part in the supercooling capability of the buds as discussed before. After deacclimation, peach floral bud loses its ability of supercooling as xylem vessel elements developed and give free access to ice to enter bud primordia (Ashworth, 1982, 1984). Presumably, after xylem continuity is established, low temperature pre-treatment would no longer facilitate supercooling, as xylem continuity already is completed. The winter of 2016-2017 was unseasonably warm and offered insufficient chilling hours for peach trees. Floral buds of ‘Flavorich’ deacclimated quite early in the last season (January 23, 2017), and had prolonged non-uniform bloom. Floral buds might have established the xylem continuity early on without advanced morphological progress. Thus, incubating floral bud at -2 °C overnight did not affect the internal water behavior in ‘Flavorich’ floral buds as we expected. On the other hand, ‘Elberta’ floral buds did not deacclimate or break. However, if this hypothesis stood true, floral buds of ‘Flavorich’ would lose supercooling ability and LTE would not occur at low temperature as we observed. But it is worth noting that

HTE was generally missing in the pre-treated DTA test for 'Flavorich' (data not shown). Therefore, this hypothesis remains to be tested.

The effect in LTE estimation after treating floral buds with low temperature overnight might alternatively be explained by the loss of moisture. Kovacs et al. (2002) performed DTA test on grape floral buds to study the effect of moisture loss during sample processing. Interestingly, 'Vignoles' and 'Norton' grape floral buds lost about 10.5% and 6.9% of their water after being excised from the stem and left at room temperature for 5 min. A 6.5% and 4.3% moisture loss of 'Vignoles' and 'Norton' buds significantly decreased their LTE temperature (P<0.001). In our study, floral buds of both cultivars normally lost 10% of buds' fresh weight after a regular DTA test, while after a DTA test with pre-treatment, weight loss was usually around 30% of buds' fresh weight (data not shown).

The correlations between LT_{50} and LTEs temperatures from pre-treated DTA were higher than correlations between LT_{50} with LTEs from regular DTA after deacclimation. Correlations between floral bud LTE measured by DTA and floral buds LT_{50} have been previously reported in various plant species. In *Prunus* spp., Quamme (1974) tested 'Elberta' and 'Siberian C' peach floral buds from November to late March and found that LTE and LT_{50} temperatures were similar, with less than 2.5 °C of difference. He further tested 10 peach varieties in winter and found good correlations between LT_{50} and LTE, r=0.880 (Quamme et al., 1975). In other *Prunus* spp., floral bud LTE temperatures were also reported to be close to LT_{50} (Andrews and Proebsting Jr., 1987; Quamme, 1974). For blueberry floral buds, correlations of LTEs to LT_{50} were high during acclimation and midwinter with r=0.85 (Biermann et al., 1979). Floral buds were

subjected to DTA test without pre-treatment, which suggested that regular DTA might be suited for cold hardiness estimation of acclimated floral buds during mid-winter. In our study, however, data collection during acclimation was not extensive. In the 2015-2016 season, floral buds were acclimated in four out of all our collection dates. In the 2016-2017 season, 'Flavorich' deacclimated early, leaving us with three dates of data available from DTA test before deacclimation. Future studies are needed to accumulate more data during acclimation.

During the acclimation process of the 2016-2017 season, both DTA tests failed to detect exotherms. During 2016 and early 2017, the southeastern U.S. and the state of Georgia was suffering from a drought which might have contributed to the failure of DTA tests to detect exotherms. Water content of floral buds at the end of 2016 were much lower than the water content at end of 2015 (Fig. 3.4), which supported this assumption. The heat of fusion released from water during the phase transition could have been too small to be sensed by a thermocouple when the bud water content was too low. Thus, exotherm events failed to be captured by DTA analyses. In this context, Biermann et al. (1979) tested floral buds from a hardy blueberry hybrid during mid-winter, and he found that cold adapted floral buds with low tissue moisture content yielded no DTA exotherms.

In summary, we explored the potential of DTA to accurately measure cold hardiness. Our results showed that the pre-treated DTA was able to overcome the inefficiency of detecting LTEs after deacclimation offering high correlations when compared to the standard artificial freezing test. DTA would allow growers to obtain cold hardiness information from samples collected in the field one day prior to the forecasted

natural frost. This method would allow our program to predict possible damages produced by upcoming frost events based on the cold hardiness information of different varieties. This will help growers to better allocate resources and decide their frost protection strategies, for example, turning on irrigation or wind machines for the night to protect cold susceptible cultivars. In the future, calibration and further refinement of our prediction models is needed for DTA to accurately predict cold hardiness of plants under natural conditions. Also, more studies are required to analyze samples that have low water content.

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Table 3.1. LT_{50} for floral buds attached to stems (5 cm and 20 cm), and LTE of regular and pre-treated DTA for 'Elberta' peach variety floral buds for winter seasons of 2015-2016 and 2016-2017.

Collection Date	Cultivar	LT_{50} (°C)	$LTE_{regular}$ (°C)[z]	Peak No.	$LTE_{pre-treated}$ (°C)	Peak No.	Bud Stage[y]	Chilling hours[x]
Season 2015-2016								
11/16/15	Elberta	-14.6 ± 5.6[w]	-16.3 ± 1.6	9[v]	-[u]	-	Tight bud	36
12/01/15	Elberta	-12.5 ± 3.3	-10.2 ± 0.8	3	-	-	Tight bud	75
01/04/16	Elberta	-13.4 ± 4.8	-13.6 ± 2	22	-	-	Tight bud	217
01/12/16	Elberta	-15.1 ± 3.1	-21.1 ± 1.6	8	-	-	Tight bud	314
01/19/16	Elberta	-15.6 ± 4.1	-16.6 ± 4.2	29	-	-	Tight bud	393
01/26/16	Elberta	-18.2 ± 3.3	-15.4 ± 2.6	27	-17.3 ± 2.9	31	Tight bud	480
02/02/16	Elberta	-15.9 ± 4	-15.6 ± 2.4	27	-18.8 ± 3	42	Tight bud	513
02/09/16	Elberta	-16.7 ± 3.9	-16.9 ± 2.4	13	-18.1 ± 3	37	Tight bud	600
02/15/16	Elberta	-16.9 ± 3.4	-17.7 ± 1.8	17	-18.9 ± 2.9	36	Tight bud	686
02/23/16	Elberta	-14.3 ± 4.5	-14.8 ± 1.7	6	-18.4 ± 0.7	29	Tight bud	713
03/01/16	Elberta	-11.1 ± 2.5	\[t]	\	-16.1 ± 2.4	25	Tight bud	760
03/07/16	Elberta	-10.3 ± 3	\	\	-13.4 ± 3.2	20	Bud swell	793
03/09/16	Elberta	-9.4 ± 3.3	\	\	-11.4 ± 3.4	9	Green bud	793
03/15/16	Elberta	-9.8 ± 1.3	\	\	-8 ± 1.3	30	Bloom	793
03/22/16	Elberta	-9.6 ± 2.4	\	\	-12.7 ± 0.7	3	Petal fall	814
03/29/16	Elberta	-7.7 ± 1.7	\	\	\	\	Petal fall	820
04/05/16	Elberta	-7.5 ± 3.5	\	\	\	\	Shuck split	822
Season 2016-2017								
10/10/16	Elberta	-13.7 ± 3	-9.1 ± 2.2	10	\	\	Tight bud	0
10/24/16	Elberta	-13.1 ± 4.1	\	\	\	\	Tight bud	5
11/07/16	Elberta	-16.3 ± 3.7	\	\	\	\	Tight bud	6
11/21/16	Elberta	-17.2 ± 3.4	\	\	\	\	Tight bud	54
12/11/16	Elberta	-19.1 ± 1	-17.9 ± 1.3	17	-19.4 ± 2.2	16	Tight bud	146
01/09/17	Elberta	-18.6 ± 2.8	\	\	-20 ± 1.9	20	Tight bud	362

01/15/17	Elberta	-17.6 ± 2.9	-17.9 ± 0.9	14	-20.1 ± 1.9	34	Tight bud	373
01/23/17	Elberta	-17.1 ± 2.2	-18.1 ± 1.7	11	-18.9 ± 2	30	Tight bud	373
01/30/17	Elberta	-17.6 ± 3.1	-17.6 ± 1.5	27	-19.3 ± 2.3	25	Tight bud	438
02/06/17	Elberta	-18.4 ± 1.9	-18.7 ± 0.8	38	-18.5 ± 3.8	39	Tight bud	470
02/13/17	Elberta	-16.5 ± 2.9	-16.6 ± 1.8	7	-19.7 ± 1.5	35	Tight bud	489
02/20/17	Elberta	-16.9 ± 2.7	-16.8 ± 2.2	32	-19.1 ± 2.2	43	Tight bud	511
02/27/17	Elberta	-16.3 ± 3.7	-15.9 ± 1.7	17	-18.6 ± 2.7	39	Tight bud	518
03/06/17	Elberta	-16.3 ± 2.3	-14.8 ± 1.8	16	-16.9 ± 3.1	35	Tight bud	536
03/13/17	Elberta	-15.6 ± 2.6	-19.3 ± 0.9	1	-19.3 ± 1.9	33	Tight bud	570
03/20/17	Elberta	-14.1 ± 4.4	-16.6 ± 1.3	11	-18.5 ± 1.4	34	Tight bud	633
03/27/17	Elberta	-15 ± 4.3	-16.8 ± 1.4	22	-17.2 ± 1.3	22	Tight bud	633

[z]LTE from regular DTA (no pretreatment) was denoted as $LTE_{regular}$, and LTE from pre-treated DTA was denoted as $LTE_{pre-treated}$.

[y]Most prominent floral bud development stage per date across samples (Horton and Johnson, 2005).

[x]Chill hour accumulation in Fort Valley, GA at each collection date based on the chilling hours model (temperature ≤ 7.2°C).

[w]Mean values ± standard deviations for LT_{50}, $LTE_{regular}$, $LTE_{pre-treated}$ were reported.

[v]A total of 48 floral buds were tested for DTA for each cultivar per date. The number of LTEs detected corresponded to these 48 buds, with the exception of the tests on 11/16/15 and 12/01/15, when 24 buds were tested.

[u]Symbol - represents dates in which DTA tests with pre-treatment were not performed.

[t]Symbol \ represents dates in which DTA failed to detect LTE.

Table 3.2. LT_{50} for floral buds attached to stems (5 cm and 20 cm), and LTE of regular and pre-treated DTA for 'Flavorich' peach variety floral buds for winter seasons of 2015-2016 and 2016-2017.

Collection Date	Cultivar	LT_{50} (°C)	$LTE_{regular}$ (°C)[z]	Peak No.	$LTE_{pre-treated}$ (°C)	Peak No.	Bud Stage[y]	Chilling hours[x]
Season 2015-2016								
11/16/15	Flavorich	-14.4 ± 2.4[w]	-12.6 ± 0.5	2[v]	-[u]	-	Tight bud	36
12/01/15	Flavorich	-12.3 ± 3.4	-13.9 ± 0.7	2	-	-	Tight bud	75
01/04/16	Flavorich	-13.5 ± 2.5	-12.6 ± 2.1	31	-	-	Tight bud	217
01/12/16	Flavorich	-15 ± 2.2	-16.2 ± 2.7	38	-	-	Tight bud	314
01/19/16	Flavorich	-14.3 ± 2.7	-11.9 ± 2.7	26	-	-	Tight bud	393
01/26/16	Flavorich	-15.2 ± 2.2	-11.7 ± 2.7	27	-20.3 ± 2.4	27	Tight bud	480
02/02/16	Flavorich	-15.7 ± 1.7	-12.4 ± 3.2	26	-14.4 ± 2.2	41	Tight bud	513
02/09/16	Flavorich	-14.6 ± 2.1	-13.2 ± 1.9	13	-14.5 ± 3.1	45	Tight bud	600
02/15/16	Flavorich	-14.8 ± 2.4	-13.8 ± 2.3	22	-17.7 ± 2.3	30	Tight bud	686
02/23/16	Flavorich	-10.7 ± 4	-12.9 ± 1.2	3	-14.4 ± 3.8	41	Tight bud	713
03/01/16	Flavorich	-9.7 ± 3.9	\[t]	\	-15 ± 2.3	15	Bud swell	760
03/07/16	Flavorich	-9.8 ± 2	\	\	-14.7 ± 1.2	12	Green bud	793
03/09/16	Flavorich	-8.8 ± 1.9	\	\	-7.7 ± 2.2	22	Pink	793
03/15/16	Flavorich	-7.9 ± 2.2	\	\	-10.2 ± 3.1	30	Shuck split	793

03/22/16	Flavorich	-8.3 ± 2.3	\	\	-14.1 ± 1.5	8	Shuck split	814
03/29/16	Flavorich	-6.3 ± 3.4	\	\	\	\	Shuck off	820
04/05/16	Flavorich	-5.2 ± 2.1	\	\	\	\	Shuck off	822
Season 2016-2017								
10/10/16	Flavorich	-15.7 ± 3.4	-9.2 ± 2.1	8	\	\	Tight bud	0
10/24/16	Flavorich	-14.7 ± 4	\	\	\	\	Tight bud	5
11/07/16	Flavorich	-17.1 ± 4.4	\	\	\	\	Tight bud	6
11/21/16	Flavorich	-18.9 ± 2.9	\	\	\	\	Tight bud	54
12/11/16	Flavorich	-18.4 ± 2.4	-17 ± 1.6	16	-21.6 ± 1.7	30	Tight bud	146
01/09/17	Flavorich	-18.2 ± 2.2	\	\	-20.3 ± 1.9	32	Tight bud	362
01/15/17	Flavorich	-18.7 ± 1.3	-17.7 ± 1.1	10	-19.9 ± 1.9	36	Tight bud	373
01/23/17	Flavorich	-16.1 ± 2.2	-16.3 ± 1.7	18	-18.8 ± 2.7	34	Tight bud	373
01/30/17	Flavorich	-16.5 ± 2.4	-16.1 ± 2	16	-17.8 ± 3.3	36	Tight bud	438
02/06/17	Flavorich	-16.4 ± 3	-17.8 ± 1.1	21	-17.2 ± 2.9	49	Tight bud	470
02/13/17	Flavorich	-15.7 ± 3.4	-17.7 ± 1.2	19	-17.1 ± 3.3	41	Tight bud	489
02/20/17	Flavorich	-14.6 ± 1.9	-17.5 ± 0.8	15	-17.4 ± 2.9	39	Bud swell	511

02/27/17	Flavorich	-12.7 ± 3.5	-15.4 ± 2.2	22	-16.1 ± 4.4	42	Bud swell	518
03/06/17	Flavorich	-10.9 ± 3.4	-15.4 ± 0.6	4	-16 ± 2	35	Bud swell	536
03/13/17	Flavorich	-9.2 ± 3.5	-15.3 ± 1.2	5	-14.4 ± 2.6	29	Green bud	570
03/20/17	Flavorich	-5.5 ± 3.9	-15.4 ± 1.8	8	-14.4 ± 1.2	16	Green bud	633
03/27/17	Flavorich	-7.1 ± 1.9	-14.6 ± 0.8	21	-14.1 ± 1.8	22	Bloom	633

[z]LTE from regular DTA (no pretreatment) was denoted as $LTE_{regular}$, and LTE from pre-treated DTA was denoted as $LTE_{pre-treated}$.

[y]Most prominent floral bud development stage per date across samples (Horton and Johnson, 2005).

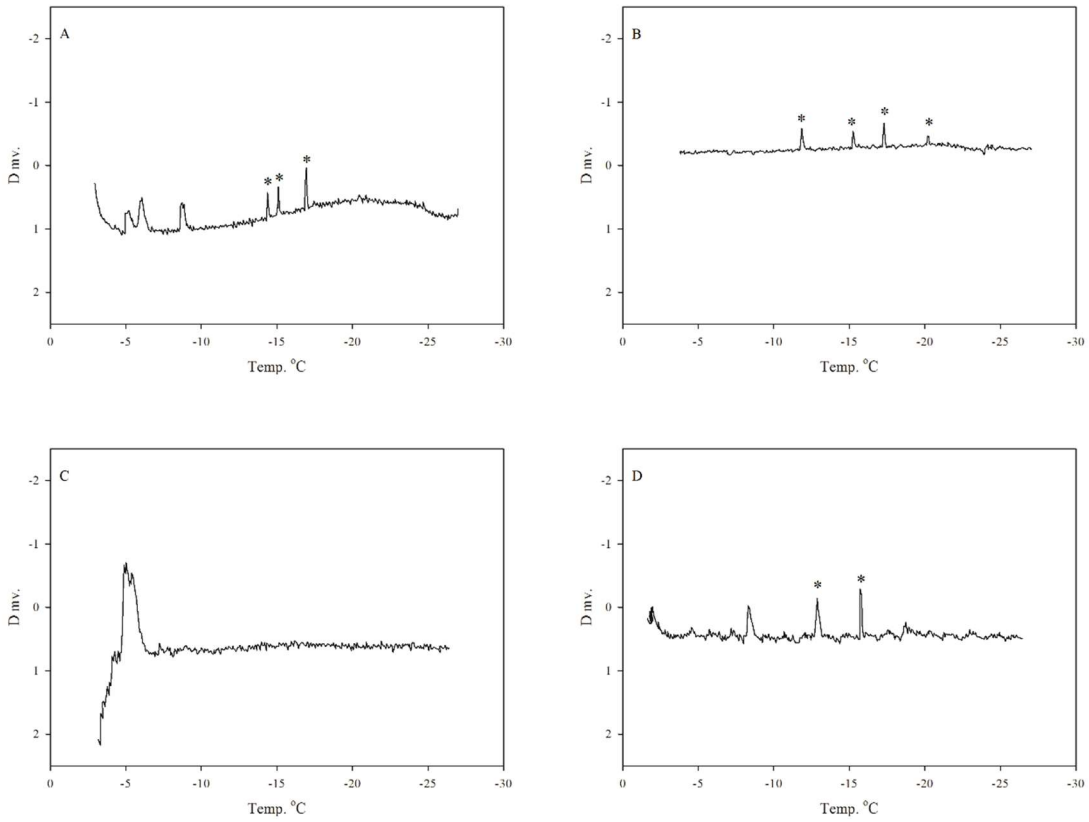
[x]Chill hour accumulation in Fort Valley, GA at each collection date based on the chilling hours model (temperature ≤ 7.2°C).

[w]Mean values ± standard deviations for LT_{50}, $LTE_{regular}$, $LTE_{pre-treated}$ were reported.

[v]A total of 48 floral buds were tested for DTA for each cultivar per date. The number of LTEs detected corresponded to these 48 buds, with the exception of tests on 11/16/15 and 12/01/15, when 24 buds were tested.

[u]Symbol - represents dates in which DTA tests with pre-treatment were not performed.

[t]Symbol \ represents dates in which DTA failed to detect LTE.


A
B
C
D
D mv.
Temp. °C
-2
-1
0
1
2
0
-5
-10
-15
-20
-25
-30

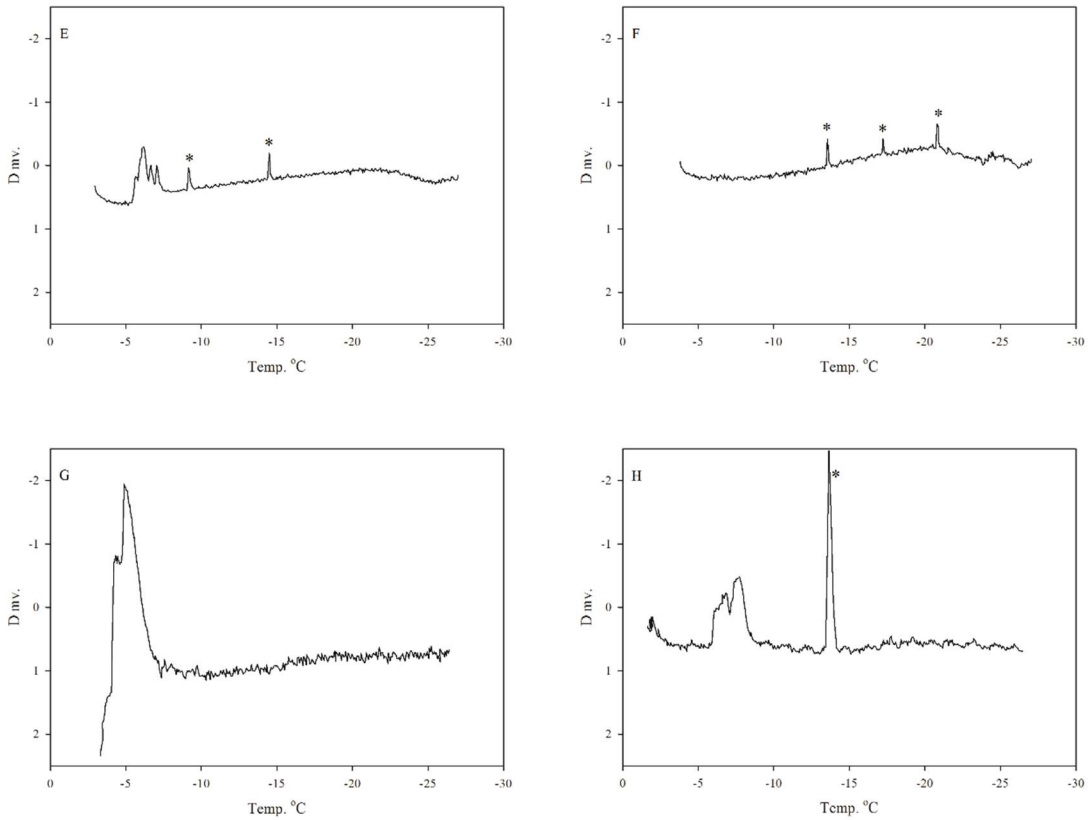


Fig. 3.1. Examples of differential thermal analyses (DTA) profiles of one thermoelectric module (TEM) during DTA tests. High temperature exotherms (HTE)s and low temperature exotherms (LTE)s can be distinguished by their shapes and locations. HTEs were observed at higher temperature (closer to positive values in the x axis), and were wider than the LTEs. DTA profiles changed when tests were performed with different cultivars, bud stages, and DTA cooling schemes (with or without pre-treatment). Acclimated floral buds of 'Elberta' (A, B) and 'Flavorich' (E, F) were collected on January 26, 2016, and tested with both "regular DTA" (A, E) and with "pre-treatment DTA" (B, F) for each variety respectively. Deacclimated floral buds of 'Elberta' (C, D) and 'Flavorich' (G, H) were collected on March 7, 2016, and similarly were subjected to both "regular DTA" (C, G) and DTA with "pre-treatment" (D, H) for each variety respectively. Symbol * denoted LTE peaks in the DTA profiles.

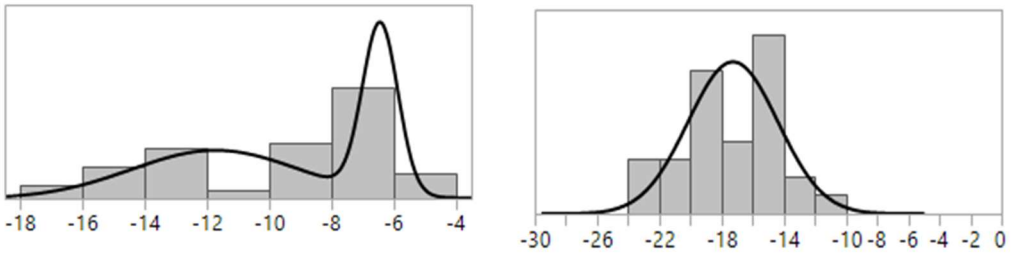
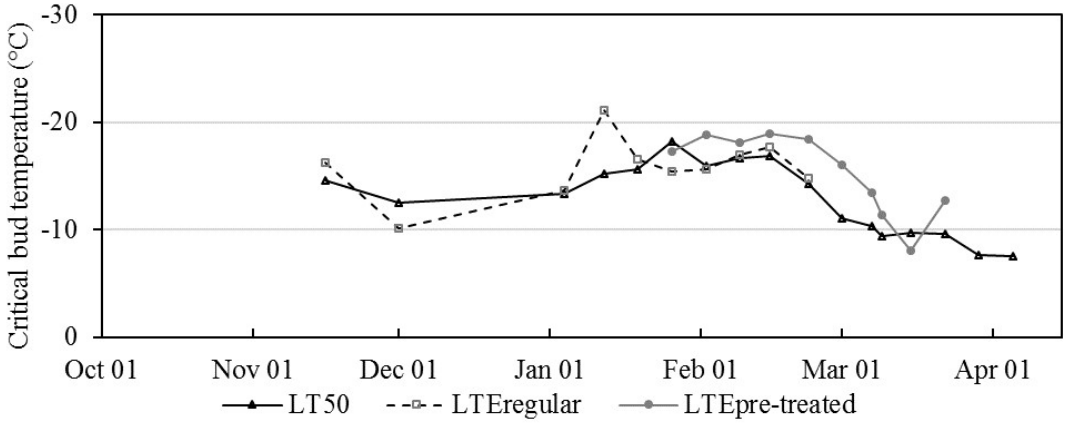
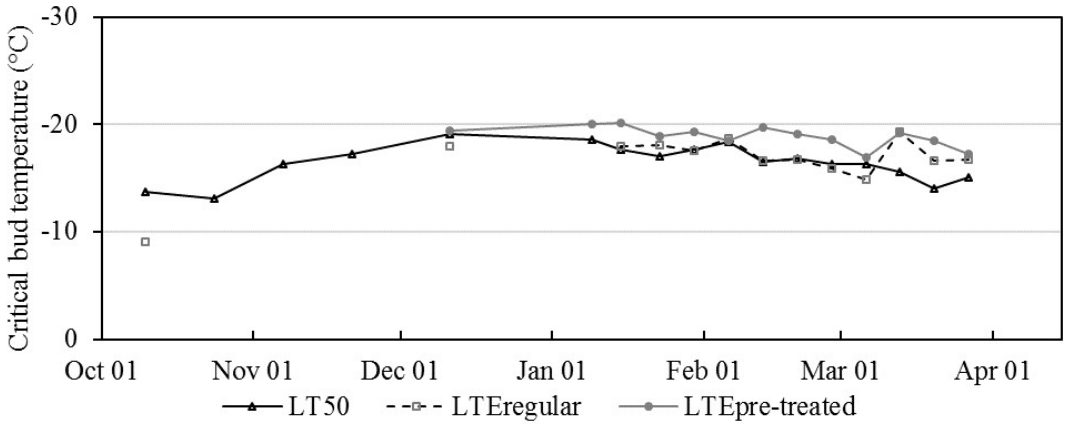


Fig. 3.2. Examples of temperature distribution of exotherm fitted by JMP Pro13. Samples were collected on January 26, 2016. Forty eight 'Flavorich' floral buds were tested for each test. Left: Normal 2 Mixture distribution of exotherms detected by pre-treated DTA. Right: Normal distribution of exotherms detected by regular DTA.

A


-30
-20
-10
0
Critical bud temperature (°C)
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
LT50
LTEregular
LTEpre-treated

B


-30
-20
-10
0
Critical bud temperature (°C)
Oct 01
Nov 01
Dec 01
Jan 01
Feb 01
Mar 01
Apr 01
LT50
LTEregular
LTEpre-treated

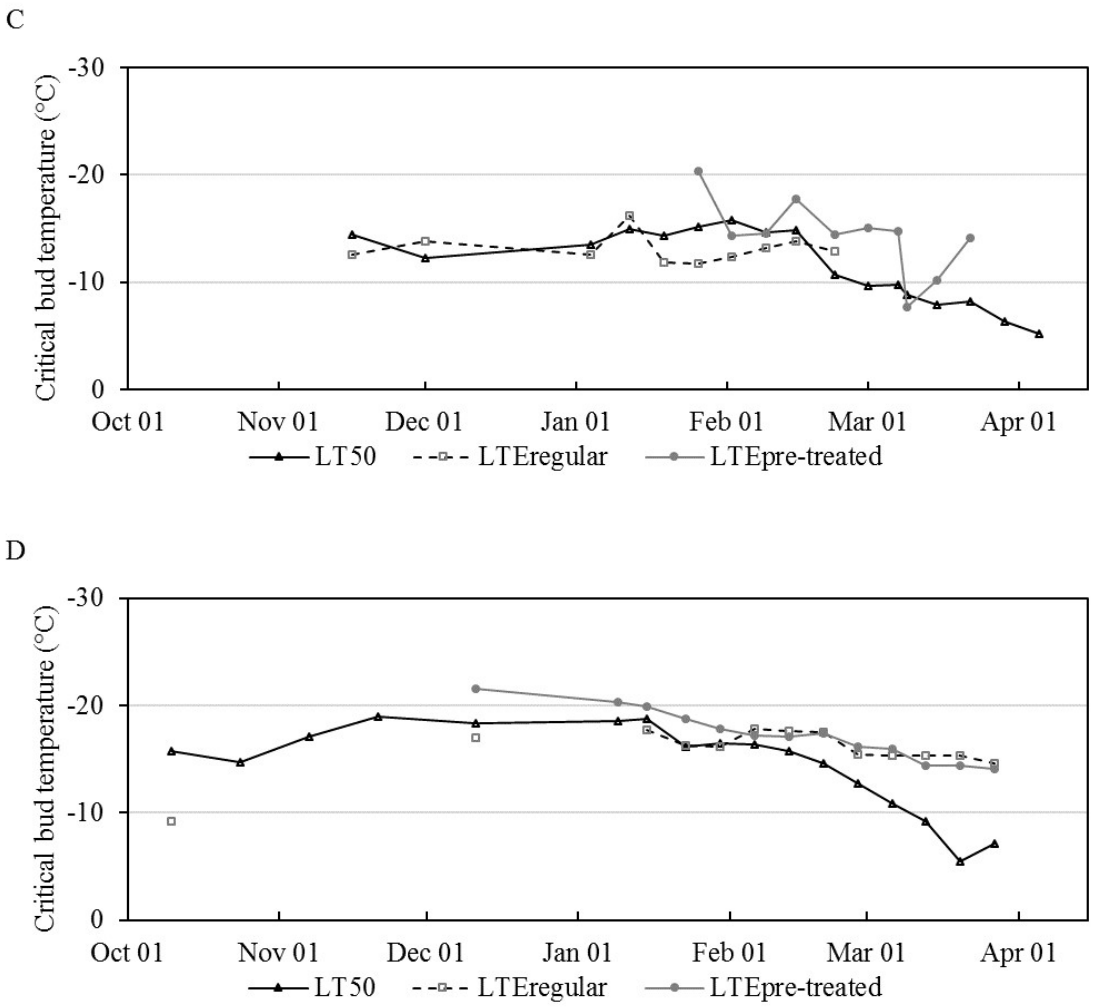


Fig. 3.3. Variation of critical bud temperature (LT_{50}) and temperatures of LTE from both DTA tests through time. Artificial freezing test and DTA were performed on 'Elberta' (A, B) and 'Flavorich' (C, D) through the winter season of 2015-2016 (A, C) and winter season of 2016-2017 (B, D). Temperatures of LTEs from regular DTA were noted as "$LTE_{regular}$" and temperature of LTEs from pre-treated DTA were noted as "$LTE_{pre\text{-}treated}$".

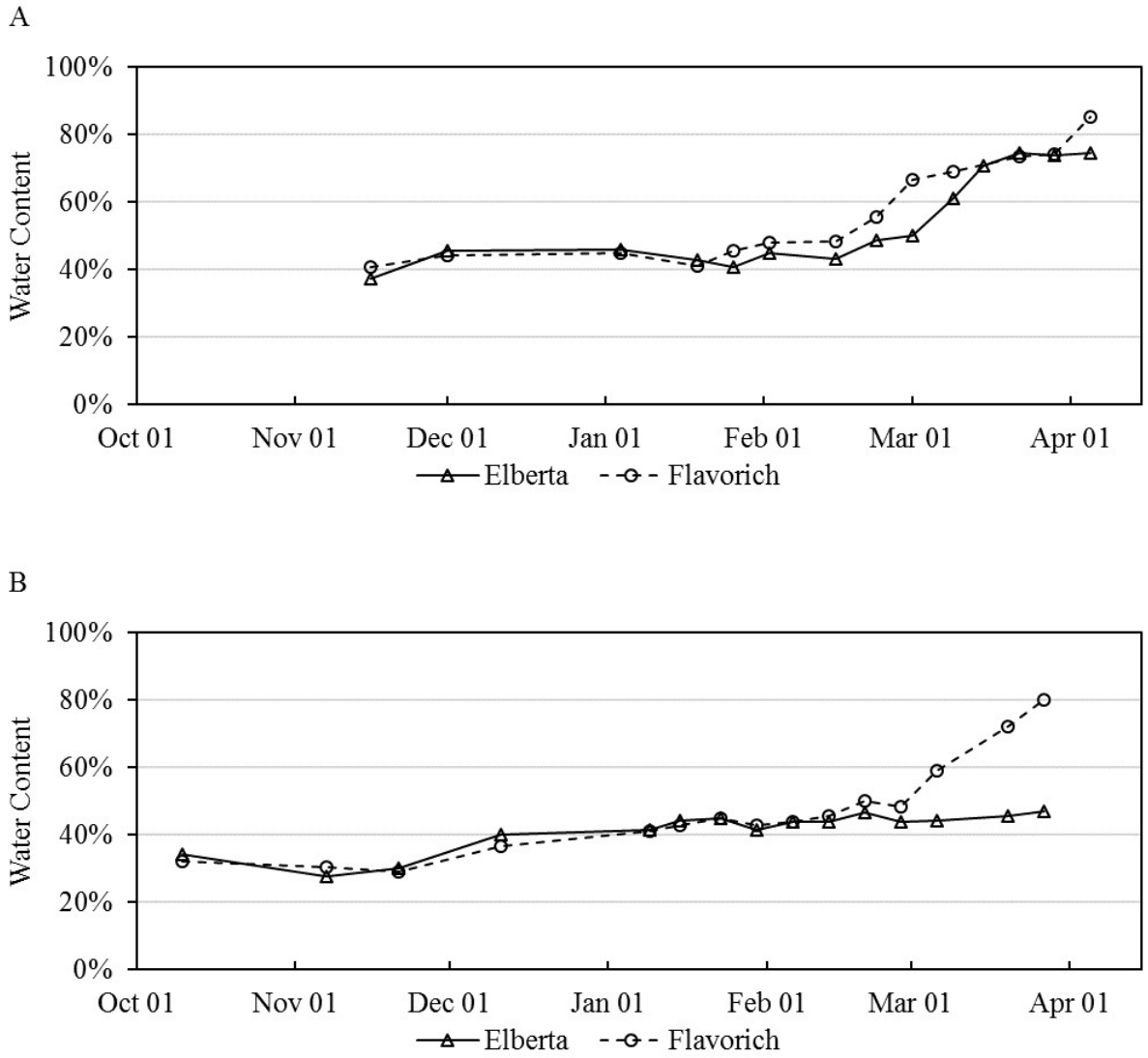


Fig. 3.4. Seasonal fluctuation of 'Elberta' and 'Flavorich' floral buds' water content in the winter season of 2015-2016 (A) and winter season of 2016-2017 (B). Bud water content was expressed as percentage of floral bud fresh weight.

CHAPTER 4

LOW TEMPERATURE DAMAGE OF PEACH FLORAL BUDS AS ASSESSED BY VITAL STAINING[1]

[1]Liu, J. et al. Submitted to *Acta Horticulturae*, 07/02/2017.

Abstract

Spring freezes in the southeast U.S. can cause damage to peach flowers after plant deacclimation. Winter, on the other hand, is usually not cold enough to produce major damage. However, understanding the magnitude of internal damage produced by freeze events in peach floral buds is of importance. The objective of this project is to recognize the susceptibility of floral bud internal structures to freeze events using a vital staining technique. Floral buds were collected in winter of 2016-2017, and treated with low temperatures ranging from 4°C to -24°C in a temperature-controlled programmable freezing chamber. Fluorescein diacetate (FDA) staining was used to identify the vital structure of floral buds that were damaged after subjected to low temperature treatments. Viable tissues stained with FDA presented a green fluorescence under 450-490 nm blue light. Cold damage was observed as a dark non-fluorescent region. Critical bud temperature that caused damage to 50% of samples (LT_{50}) based on the fluorescence was calculated for each internal floral structure. For floral buds collected during dormant bud stage (tight bud) on November 21, 2016, the flower pistils were short, ovaries were not swollen, and stamens were immature. We found that during this time, young pistils tended to show cold damage first, with a LT_{50} = -15.4 °C, followed by anthers (LT_{50}= -16.8 °C), and then petals and sepals (LT_{50} = -17.2 °C). As the floral buds further developed in the field in spring of 2017, internal structures in the floral buds developed with cold susceptibility of these structures being variable. Stamen tended to be most cold resistant (LT_{50} = -15.2 °C), and pistil and corolla were cold susceptible (LT_{50} = -12.0 °C and -11.0 °C, respectively). This study offers important information on cold damage associated with specific floral bud internal structures.

Introduction

Georgia ranks third in peach production in the United States with 10,000 acres of bearing peach and 40,600 tons of production in 2015 (USDA, 2016). Freezing damage counts as one of the major causes of yield loss in peach production in Georgia. Peach floral buds are the most sensitive organ to low temperatures in a peach plant, especially after deacclimation, when buds have lost their cold resistance. Freezes, subsequently, are most devastating to peach yield after bud deacclimation and bud swell.

Within a floral bud, different structures exhibit different cold susceptibilities. In a study of highbush blueberry (*Vaccinium corymbosum* L.), cold susceptibilities were determined for different structures and listed in order from most cold vulnerable to cold hardiest as follow: corolla, filament, anther, style, exterior ovary, stigma, ovules, interior ovary, and placenta (Rowland et al., 2013). Similar cold resistance differences in rabbiteye blueberry (*Vaccinium virgatum* Aiton) floral organs were also observed, with corollas being the most sensitive, ovaries being the hardiest, and styles falling in between by using visual evaluation (NeSmith et al., 1999). In the case of peach, it was determined that before bud swell, floral buds froze as a whole unit without an intermediate form between alive buds and dead buds being observed (Quamme, 1974). After bud swell, however, the peach floral bud parts presented an order of cold hardiness from the most susceptible to the most resistant to cold as follows: pistil, anthers, corolla, calyx, and pedicel (Quamme, 1974). However, peach floral buds at a more advanced stage (close to bloom) under field conditions presented more damage variation when treated with different low temperature treatments (Oberle, 1957). In this same context, a field observation after a natural freeze noted different forms of cold damage. Some flowers

opened with pistils aborted due to cold damage, some had normally developing pistils but had no petals nor stamens, while some other flowers were intact (Oberle, 1957).

It is reasonable to assume that damage to pistils would surely lead to failure of fruit set for peach and blueberry, but how specific damage to floral organs affects fruiting is unclear. Many studies had aimed to identify the structures in floral buds that are critical to fruit set in blueberries (Gupton, 1983, NeSmith et al., 1999). Five rabbiteye cultivars were studied for cold damage after a natural freeze (Gupton, 1983). In this study, although the percentage of flowers that failed to set fruit correlated with the percentage of flowers displaying corolla damage, failure of fruit set was more likely attributed to pistil damage rather than the decreased attraction to pollinators. Some floral buds were hand-pollinated after the freeze, but no significant differences of fruit set were shown between hand-pollinated flowers and open-pollinated flowers. Similarly, NeSmith et al. (1999) noted that, after exposing open flowers or floral buds about to open (stage 5 - individual flowers distinctly separated, corollas unexpanded and closed, or stage 6 – corollas completely expanded and open) to -1 °C for 1 h, a decline of fruit set occurred down to 20%. The authors also observed that bee pollination also declined even without any visual damage to any structure at this temperature. They concluded that there was a 'hidden damage' that was unable to be picked up by visual examination (NeSmith et al., 1999). Therefore, more accurate methods are needed to identify cold damage at the floral organ level.

Fluorescein diacetate (FDA) is a type of fluorogenic ester with cell permeability. It can work as a precise probe for cell viability. FDA itself is not fluorescent, but its hydrolyzate, fluorescein, has fluorescence ability. FDA entering a viable plant cell is

hydrolyzed by intracellular esterases, emitting a fluorescent signal which indicates cell viability. Dead cells, on the other hand, do not possess such enzymatic activity and show no fluorescent signal (Jones et al., 2016). Previous researchers had successfully utilized FDA to assess freezing injury of leaves of alpine plants (Yamori et al., 2005). FDA can be used as an accurate and precise viability probe for plant tissues. The objective of our study is to characterize the susceptibility of the internal structures of peach floral buds to freeze events using a vital staining technique.

Materials and Methods

Plant material

Peach, *Prunus persica* (L.) Batsch, 'Elberta' plant tissues were collected on November 21, 2016; February 6, 2017; and March 27, 2017. One-year old twigs were collected from fruit- bearing peach trees planted in 2010 in a commercial orchard in Fort Valley, Georgia, USA. Twigs with attached floral buds were wrapped in plastic bags and transported to the University of Georgia, Griffin Campus, Griffin, Georgia, USA in a cooler with ice. Twigs were then cut into 20 cm pieces with floral buds still attached and were randomly divided into nine groups. Each group contained four twigs which were wrapped with damp Kimwipes (Kimberly-Clark, USA) and sealed in plastic bags to prevent drying out. One group of samples was used as control and the other groups were assigned to different temperature treatments ranging from -3 °C to -24 °C with an interval of -3 °C between treatments. Samples were then stored in a refrigerator at 4 °C for less than 24 h prior to analyses.

Artificial Low Temperature Treatment

Samples were treated within a temperature-controlled programmable freezing chamber (Temperature and humidity chamber PR-3FPH, Tabai ESPEC, Japan), except for the control group, which was left at 4 °C. Samples within bags were hung on a wood rack with metal rods in the freezing chamber. Temperature inside the freezing chamber was held at -2 °C overnight, then decreased at a rate of 4 °C h^{-1}. Samples for each group were removed from the freezing chamber quickly when their designated temperature treatments were reached, and transferred back to 4 °C.

Cold hardiness Evaluation

1. Visual Evaluation

Samples were incubated at 4 °C for about seven days after freezing treatments. During the 7-day period of recovery, cold injured tissue oxidized and developed a yellow to brown discoloration at the injured region. Floral buds were bisected under Meiji EMZ-5TR Stereo Microscope (Meiji, Japan) and observed for browning. Buds were rated as alive when the whole bud was green without discoloration. On the other hand, when brown color was observed inside of the floral buds, the buds were rated as dead. Eight buds were examined for each temperature treatment group, and a total 80 buds were evaluated in each date. A series rate was generated and the critical temperatures that killed 50% buds (LT_{50}) were calculated by fitting a logistic function using the ratings.

2. Staining Method

Further survival of floral buds and floral bud parts were studied using the in vivo FDA staining method. Since our preliminary data suggested that incubation time did not affect FDA staining result (data not shown), buds were also incubated at 4 °C for seven days to be consistent with the visual evaluation method. FDA stock solution was made by dissolving 1 mg FDA (Fluorescein diacetate 97%, catalog No. AAB2446606, Alfa Aesar, USA) in 1 mL acetone, and stored at -20 °C. FDA working solution was made by mixing 10 µL FDA stock solution with 1 mL water, with a final concentration of 10 µg mL^{-1}. The FDA working solution was made fresh prior to each analysis and stored in a cold and dark setting for up to 2 h. Since bud scales excessively absorbed FDA and prevented FDA from entering floral bud organs, bud scales were peeled off from floral buds to expose developing reproductive tissues. A thin slice of the floral primordium tissue was removed from the sample by freehand sectioning using two-double edge blades attached together. A total of eight buds in each freezing treatment group were analyzed. The tissue slice was placed on a glass microscope slide and few drops of 10 µg mL^{-1} FDA solution were added. Samples were covered with a coverslip. Tissues were incubated in the dark for about 15 min, and observed under a Leitz Fluovert Inverted Microscope (Leica, Germany) with a Leitz I 2/3filter block (excitation filter: band pass 450-490 nm; barrier filter: long pass 515 nm) (Leica, Germany). Tissues or flower parts that fluoresced green under the inverted microscope were rated as alive. Tissues or flower parts that were dark and did not fluoresce were considered cold damaged. Photos were taken with a AmScope Microscope Digital Camera MU1000 (AmScope, USA) under a 200x magnification. A

rate of tissue viability similar to the visual evaluation rating was obtained and LT_{50} was calculated for each floral part.

Data Analysis

Data were analyzed with JMP Pro 13 (SAS Institute Inc., United States). Calculation of LT_{50} was done by fitting logistic function to ratings of whole floral bud or floral bud parts. Student's t-test was performed to determine difference in cold hardiness among floral bud parts and collection dates.

Results and Discussion

Floral Bud Morphology

Evaluation of buds collected on November 21, 2016, revealed that the pistils were short, ovaries were not swollen, and immature, bean-shaped anthers were directly attached to the second whorl (corolla) with filaments not developed (Fig. 4.1). After February 6, 2017, floral buds were bigger and had elongated pistils with a clearly defined shape. The ovary started swelling as well, and a minute cavity where ovules were going to form inside of the developing ovary was visible (Fig. 4.3). For stamens, filaments started to develop and elongate as clusters. Additionally, anthers grew bigger with their two anther lobes visible. Petals also expanded as compared with those of floral buds from the first test (Fig. 4.3). Floral buds collected March 27, 2017, showed little further development, though some petals expanded a little.

The winter of 2016-2017 was an extraordinarily warm winter. The chilling requirement of 'Elberta' peach variety is 850 chill-hours. Actual chilling hours

accumulated at Fort Valley, Georgia, USA were 54 chill-hours on November 21, 2016. At February 6, 2017 and March 27, 2017, 470 and 633 chill-hours were accumulated at Fort Valley, Georgia, USA, respectively. Floral buds of 'Elberta' were at the tight bud stage through this whole study. At the last sampling date, floral buds of 'Elberta' were bigger in size, however, they did not yet reach bud swell stage. The lack of chilling contributed to the slow floral bud development progression.

Cold Hardiness

The LT_{50} of whole buds was calculated from the visual evaluations; the LT_{50} of floral bud parts (pistil, stamen, and corolla) was determined from the FDA staining method (Table 4.1). Student's t-test detected significant differences of LT_{50} among parts (pistil, stamen, and corolla) in floral buds and whole buds during the second test date (Table 4.1), similar to what Quamme (1974) observed. Of individual floral structures, the LT_{50} of pistil and stamen showed a trend of increasing toward warmer temperatures with time, but did not show significant differences between dates. Though not significant, the corollas lost hardiness from -17.2 °C to -11.0 °C from the second test to the third (Table 4.1).

During the tight bud stage on November 21, 2016, the LT_{50} of all floral bud parts were close, although pistils tended to be the least hardy (Table 4.1). This was consistent with the hypothesis of Quamme (1974) that all bud structures freeze as a unit. For the last two test dates, when floral bud development progressed more, differences between LT_{50} of the different floral parts, although not statistically different, were further apart by their values (Table 4.1, Fig. 4.4, Fig. 4.5). In the February 6th evaluation, cold susceptibility of

pistil, anthers, and corolla fell into the exactly same order as previously reported by Quamme (1974).

From the first sampling date to the second sampling date, the LT_{50} of the pistil increased to a higher temperature, indicating loss of cold hardiness of the floral bud pistil. Furthermore, pistils developed and were more than twice as long visually as pistils of the first date (Fig. 4.1 and Fig. 4.3). On the first sampling date, pistils had just started elongation and anthers were detached from petals. On the third sampling date, the LT_{50} of the corolla was -11.0 °C, which is more than 6 °C higher compared with the second sampling date. This may indicate an order of deacclimation as the pistil started to lose cold hardiness first, followed by the corolla. Stamens did not show an obvious loss of cold hardiness through winter. Unfortunately, until the last sampling date, 'Elberta' floral buds were still tight, and had not progressed to bud swell. Beyond bud swell, morphology and cold hardiness changes within floral buds requires further study. These results revealed an order of deacclimation time of different structures within a peach floral bud. However, it would be important to conduct further experiments since statistical differences through the different collection dates were not detected in our study.

The LT_{50} estimated by visually evaluating whole floral buds tended to yield less conservative results when compared to LT_{50} of floral bud parts (Table 4.1). In a study of rabbiteye blueberry, NeSmith et al. (1999) noted that, after exposing open flowers or floral buds about to open to -1 °C for 1 h, a decline of fruit set down to 20% occurred, with a reduction of bee pollination even without any visual damage to any structure at this temperature. Therefore, he concluded that there was 'hidden damage' that were unable to be recognized by visual examination (NeSmith et al., 1999). FDA may

overcome the inaccuracy of visual examination. The FDA staining method is also a fast evaluation method. Our preliminary study suggested no differences in FDA analyses for buds at different recovery time (data not shown). The recovery period after the artificial freezing treatment could be shortened for future studies. Therefore, the FDA evaluation method can serve as an alternative to the visual evaluation method (Yamori et al., 2005).

FDA has some disadvantages, as do any methods for cold hardiness evaluation (Takeda et al., 1993). Fluorescence was still observed in the tissues of black currant floral buds after treatment with a lethal temperature, and was observed in incubation medium later. This fluorescence may be caused by remaining esterase activity of dead plant cells, as Takeda (1993) suggested. In our study, similar phenomenon were observed. Takeda et al. (1993) used FDA working solution that had a concentration five times the concentration used in this study. This issue may be resolved by modifying the concentration of FDA.

In this study, we utilized an FDA staining technique to study cold hardiness of interior structures of peach floral buds. We confirmed the hypothesis of Quamme (1974) that before bud swell, the floral bud freezes and is damaged as a whole unit, since the LT_{50} of floral bud parts are close to each other (Table 4.1) and partial damage of floral buds was rarely observed at this stage (Fig. 4.2). At later development stages, floral bud parts began to differentiate their cold hardiness levels. The pistil was among the first to lose cold hardiness, followed by the corolla. The stamens maintained cold hardiness until the end of this study due to the lack of chill in the exceptional warm winter of 2016-2017 of the southeastern U.S. We also compared the effectiveness of an FDA staining method to evaluate cold injury with visual evaluation. We determined that FDA staining was

more sensitive to capture cold injuries that would otherwise elude visual examination. FDA staining also provides potential for faster evaluation.

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Table 4.1. LT_{50} determined for peach floral buds for three collection dates. LT_{50} of the whole floral bud was calculated from visual evaluation. The LT_{50} of floral bud parts was calculated from the FDA staining method.

LT_{50}(C)	**November 21, 2016**	**February 6, 2017**	**March 27, 2017**
Pistil	-15.4	-12.7 A[y]	-12.0
Stamen	-16.8	-15.4 AB	-15.2
Corolla	-17.2b[z]	-17.2bB	-11.0a
Whole bud	-17.2ab	-18.4bB	-15.0a

[z]Different lowercase letters within a row represent statistically significant differences (P<0.05) for LT_{50} of different sampling date as determined by Student's t-test of same part or whole bud.

[y]Different uppercase letters within a column represent statistically significant differences (P<0.05) for LT_{50} as determined by Student's t-test within sampling date.

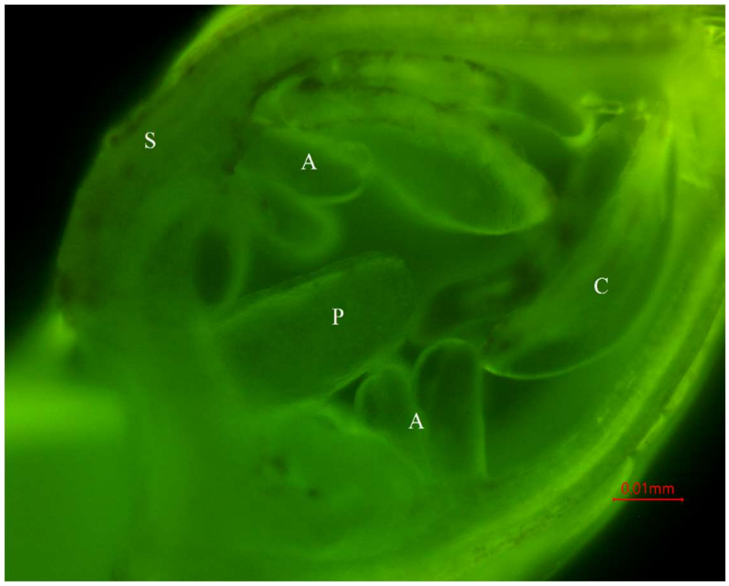

Fig. 4.1. Microscopic photo taken of alive ‘Elberta’ floral buds under UV light. Buds were collected on November 21, 2016. Young pistil, stamen and corolla were visible, all parts exhibited green fluorescence. P: pistil, A: anther, C: petal and S: sepal.

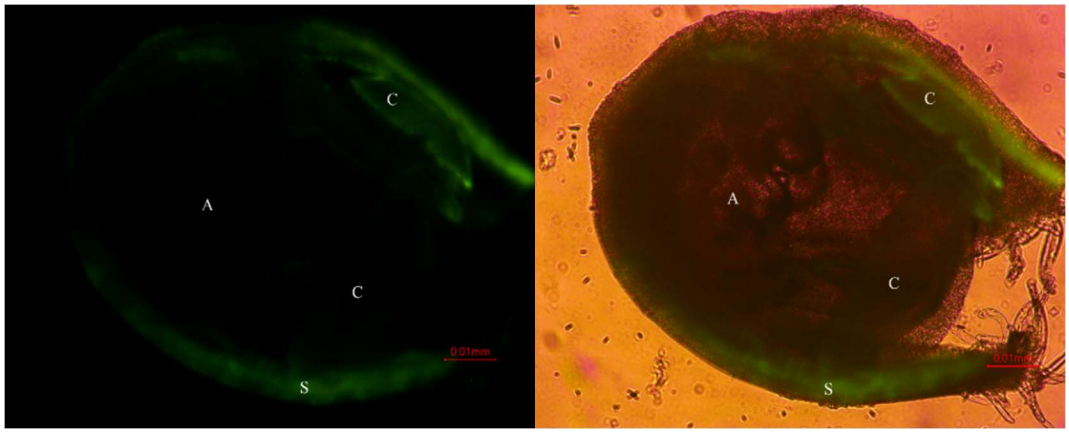

Fig. 4.2. Microscopic photo taken of dead ‘Elberta’ floral buds under UV light (left) and UV light overlaid with bright field (right). Buds were collected on November 21, 2016 and were treated with -15 °C. All parts of the bud, except for sepal and some petal tissue, did not show fluorescence, thus were considered dead. C: petal, A: anther and S: sepal.

Fig. 4.3. Microscopic photo taken of alive ‘Elberta’ floral buds under UV light. Buds were collected on February 6, 2017, and belonged to the control (no freezing) group. Bud size exceeded the viewing field of camera. The carpel was dissected to expose the inside of the developing ovary. Developing anthers and filaments were visible around the carpel. All parts exhibited green fluorescence. P: pistil, O: developing ovary and A: anther.

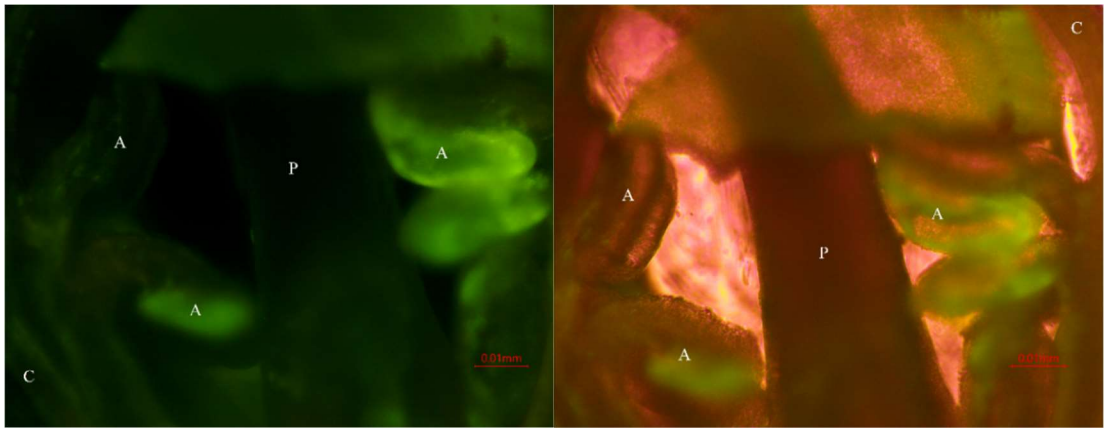

Fig. 4.4. Microscopic photo taken of dead ‘Elberta’ floral buds under UV light (left) and same bud under overlaid dark field and bright field (right). Buds were collected on February 6, 2017 and were treated with -15 °C. Dead carpel without fluorescence was visible in the center of photo. Sections of petal in the corner of photo did not fluoresce either. Some anthers still exhibited green fluorescence. P: pistil, C: petal and A: anther.

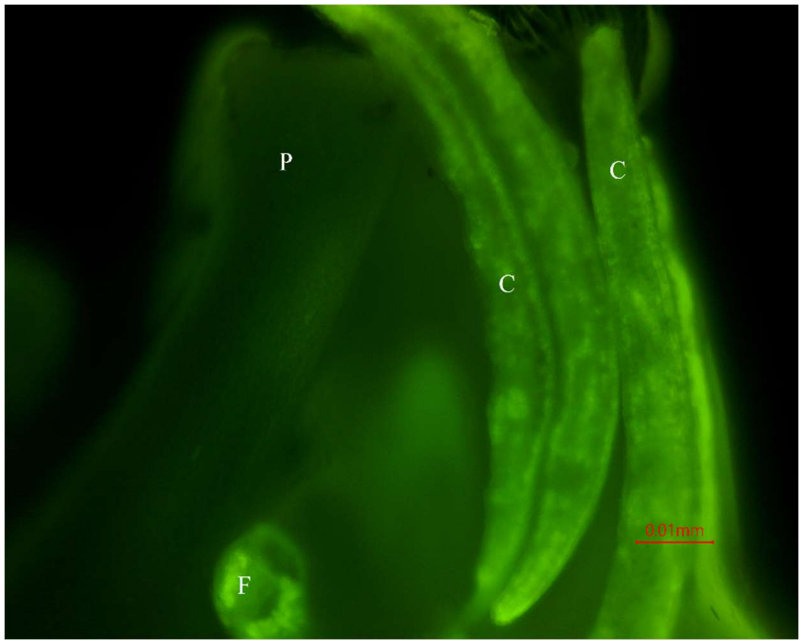

Fig. 4.5. Microscopic photo taken of dead ‘Elberta’ floral buds under UV light. Buds were collected on February 6, 2017 and were treated with -21 °C. Dead carpel without fluorescence was visible on the left side of photo. Filament and petals still exhibited green fluorescence. P: pistil; F: filament, exposing the cut phase; and C: petal.

CHAPTER 5

CONCLUSIONS

Freezes are major natural weather events that affect commercial peach production in Georgia. It is important to timely and accurately monitor cold hardiness of peach trees. With such information, peach growers can be notified and take actions prior to freezes that could cause major damages. This study was devoted to test and to improve different methods used to measure cold hardiness of peach plants and to provide a better understanding of the freezing process in peach.

Artificial freezing test is the standard method to measure cold hardiness of plant tissues and has been widely used. Artificial freezing test is featured by its accuracy. It approximates the freezing process and the freezing damage in field condition well as it evaluates directly plant tissues for cold injury. Yet, artificial freezing test is not immune to influence of test conditions. The critical temperature of peach floral buds after swell have been previously reported by Ballard, Proebsting Jr. et al. (1999) in Washington State. Information of peach cold hardiness in Georgia, however, is generally missing. We, therefore, explored how different experimental conditions affect cold hardiness determination of artificial freezing test and developed reference temperatures for Georgia peach production based on our optimized freezing test protocol (Chapter 2). We found that bud excision resulted in an increase of the LT_{50} temperature of floral buds as evaluated using the artificial freezing test, which means using the artificial freezing test

with excised floral buds (without stem tissue) tends to yield more conservative results. Additionally, we determined that using floral buds still attached to stems of different lengths, either 5 cm or 20 cm, did not significantly influenced the results of the artificial freezing test. Therefore, stems of 5 cm are recommended as sampling type for artificial freezing test in our conditions to achieve the most effective sample use and to accurately measure cold hardiness.

Artificial freezing tests have their drawbacks. It required a week of time to get samples to oxidize and discolored consistently to produce a meaningful evaluation and rating (LT_{50}). A faster method of measuring cold hardiness is needed. Differential thermal analysis (DTA), featured by its convenience, objectiveness and efficiency, has great potential. However, DTA is also argued as unreliable for its sensitivity of test conditions. Our goal was to standardize test protocol to achieve meaningful results with DTA (Chapter 3). For DTA, alternative cooling schemes were tested. The cooling scheme consisting of an overnight -2 °C incubation before start cooling at -4 $°C \cdot h^{-1}$ increased the number of LTEs detected through DTA (as compared with regular DTA without pre-treatment incubation). It also resulted in an improved correlation between temperatures of the LTEs and cold hardiness values obtained using the standard artificial freezing test.

Better understanding of freezing process within plants facilitate better interpreting peach floral bud DTA profiles. For peach, Quamme (1974) was the first to observe low temperature exotherms in flower buds. Quamme (1974) noted that a dormant bud exhibit only one LTE, but acclimated buds had several LTEs. He further proposed that all the tissue present in a dormant bud froze suddenly at the same temperature, with one single

exotherm detected by DTA. Right when buds began to swell, Quamme (1974) suggested cold hardiness of parts in the flower primordia differentiated and sudden freezes of different parts produced multiple LTEs. This hypothesis still reminds to be tested. However, based on our results, the numbers of LTEs rarely exceeded the numbers of buds that were tested at all stages, with one exception. In addition, high temperature exotherms (HTE) are induced by freezing events in bud scale and bud axis, and are not thought to caused direct damage to floral buds. Those freezing events, however, cause dehydration of flower primordia (dehydration stress) since ice located in the bud scale and axis have a low water potential and draw water out of the flower primordia. This dehydration stress can be lethal. Temperatures when HTE occur may not be lethal to buds, but lethal dehydration might happen few Celsius degrees following HTEs. It reminds to be determined if temperature where LTE occurs is really the critical temperature that causes damage to flower buds. From our result, although LTE temperatures highly correlates with LT_{50} calculated from artificial freezing test that reflect temperature of freezing injury, LTE temperatures tended to be lower than LT_{50}. Better understanding of these differences can help further refine the accuracy of DTA as a cold hardiness measurement.

Microscopic evaluation (Chapter 4) was carried out to better understand the relationships between the exotherms detected by DTA and the bud damage present after freezing. We confirmed the observation that tissues in tight bud stage freeze suddenly at the same temperature. Also, we established that the tissues within a flower bud have a different cold hardiness when were about to swell. However, the efforts to link exotherms with injury of individual parts in buds were not all that successful. No clear pattern was

observed between distribution of HTE/LTE temperatures and critical temperature of individual structures based on the microscopic analyses for floral buds.

In conclusion, statistical analysis suggested DTA was indeed a fast and reliable cold hardiness measuring method as compared to artificial freezing tests. Cold hardiness data would be available within hours with the utilization of DTA. Its capacity of measuring a large number of samples at the same time also makes it possible to provide site and cultivar specific data. DTA can help growers with their frost protection strategies in order to avoid cold damage to peach plants.

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